RICHTER, T A

SEASONALITY OF REPRODUCTION IN UNGULATES: THE EFFECT OF PHOTOPERIOD ON REPRODUCTION IN A SEASONAL BREEDER, THE SHEEP *OVIS ARIES* AND A PUTATIVE ASEASONAL BREEDER, THE SPRINGBOK *ANTIDORCAS MURSUPIALIS*

MSc UP 1998

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Seasonality of reproduction in ungulates: the effect of photoperiod on reproduction in a seasonal breeder, the sheep (*Ovis aries*) and a putative aseasonal breeder, the springbok (*Antidorcas marsupialis*)

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Abstract

I investigated reproduction in springbok (*Antidorcas marsupialis*) to determine whether the basic physiological system that underpins seasonal reproduction exists in this putatively aseasonal ungulate. In addition, I examined the transition from reproductive quiescence to activity in female mammals using the sheep (*Ovis aries*) as a model species. My analysis of reproduction in wild and captive springbok populations suggested that springbok, unlike seasonal breeders, do not use photoperiod or other climatic variables to cue reproduction. This was reinforced by the finding that the reproductive characteristics of a captive herd of springbok ewes are not the same as typical seasonal breeders. However, the pineal-melatonin system of springbok functions in the same way as in photoperiodic species. Thus, I suggest that springbok ignore photoperiodic information that could potentially be used to cue reproduction, which suggests that the physiological basis of aseasonality in springbok lies downstream from the pineal-melatonin system. The results of the investigation of the sheep suggested that the neurotransmitters that have been implicated in the regulation of reproduction might have differential roles that are determined by the reproductive state of an animal. The results supported the contention that there is a complex system of interneurones that link melatonin to the modulation of gonadotrophin secretion. In conclusion, the physiological system that regulates reproduction in ungulates is complex but nevertheless seems to be fundamentally similar in

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seasonal and aseasonal species. Thus, it seems that aseasonal breeders may not be physiologically bound to reproduce aseasonally, but reproduce in the manner that they do because it is an adaptive advantage.

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List of Abbreviations

Abbreviation	Description
∅	diameter
5-HIAA	5-hydroxyindoleacetic acid
5-HT	5-hydroxytryptamine
Ab	antibody
Ag	antigen
ANCOVA	analysis of co-variance
ANOVA	analysis of variance
Asp	aspartate
CSF	cerebrospinal fluid
CV	coefficient of variation
D	dark
DA	dopamine
DOPAC	dihydroxyphenylacetic acid
GABA	γ-aminobutyric acid
Glu	glutamate
HPLC	high pressure liquid chromatography
ID	inside diameter
L	light
LH	luteinizing hormone
LHRH	luteinizing hormone releasing hormone
NA	noradrenaline
OD	outside diameter
PBS	phosphate buffered saline
s	standard deviation
s'	degree of birth restriction
SEM	standard error of the mean

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Chapter 1

Introduction

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The fitness of an individual depends on the number of offspring it produces during its lifetime that survive to reproduce themselves (Price 1995). Hence, organisms evolve adaptations that maximise their individual fitness through maximising the probability that their offspring will survive to reach reproductive maturity. Although the reproductive adaptations that have evolved to realise this goal are numerous and diverse, they are all constrained by physiological, evolutionary and environmental factors.

The manner in which an animal behaves depends fundamentally on its genetic constitution, upon which natural selection acts. Consequently, life history traits are relatively inflexible, irrespective of the taxonomic designation of an organism. The taxonomic affiliation of an organism determines the manner of, and extent to which, reproduction is restricted by its physiology. For example, an important physiological factor that needs to be considered when examining the reproductive behaviour of female mammals is the length of gestation. Gestation length is strongly correlated with body size in mammals; larger ungulates have longer gestation periods (Figure 1). The length of gestation can vary between individuals and species but is usually constrained within narrow limits (Berger 1991). Thus, mammals need to account for the length of gestation so that the birth of young takes place in a particular season.

The environment is an important determinant of the behavioural and physiological traits of animals. Environmental forces that act upon reproduction operate at two levels, namely the ultimate and the proximate (Baker 1938). Ultimate factors are important in the long-term, evolutionary sense. Proximate factors provide immediate cueing for the onset and cessation of reproductive activity. Seen from a purely ultimate perspective, most reproductive systems reflect variation in a complex of interacting dietary and climatic factors, specifically food, rainfall and temperature (Bronson 1985). All physiological processes, including reproduction, are limited by the amount of available food. Rainfall and temperature in turn determine the amount of food, while temperature can also influence reproductive function directly. In addition to these factors, competition for resources is often influential (Fraser Darling 1938, Ims 1987).

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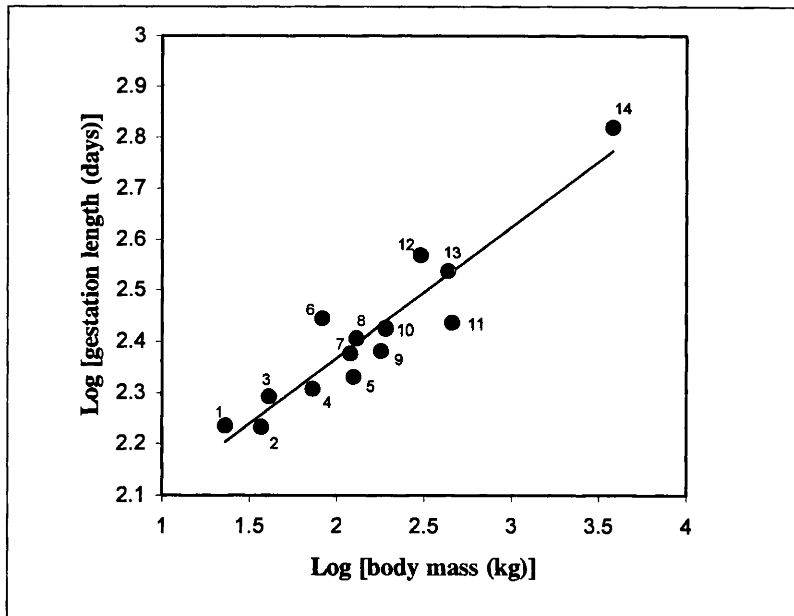

Figure 1. Relationship between body mass and gestation length for 14 species of southern African antelope. For the regression line, $y = 0.26x + 1.85$, $r^2 = 0.88$, $p < 0.05$. 1, dorcas gazelle (*Gazella dorcas*), Dittrich (1972). 2, springbok (*Antidorcas marsupialis*), Heinroth (1908), Smithers (1971). 3, impala (*Aepyceros melampus*), Fairall (1968), Smithers & Wilson (1979). 4, dama gazelle (*Gazella dama*), Antonius (1932). 5, waterbuck (*Kobus ellipsiprymnus*), Heinroth (1908), Spinage (1982). 6, tsessebe (*Damaliscus lunatus*), Dekeyser (1955), Child *et al.* (1972). 7, red hartebeest (*Alcelaphus buselaphus*), Smithers (1971), Dittrich (1972). 8, black wildebeest (*Connochaetes gnou*), Smithers (1971), Von Richter (1971), Skinner & Smithers (1990). 9, blue wildebeest (*Connochaetes taurinus*), Watson (1969). 10, sable (*Hippotragus niger*), Dittrich (1972), Happold (1987). 11, eland (*Taurotragus oryx*), Posselt (1963), Skinner & Van Zyl (1969). 12, burchell's zebra (*Equus burchelli*), Wackernagel (1964), Smuts (1974). 13, African buffalo (*Syncerus cafer*), Vidler *et al.* (1963), Patterson (1979). 14, African elephant (*Loxodonta africana*), Perry (1953), Robertson-Bullock (1962).

In many regions, harsh climatic conditions prevail seasonally and animals that restrict reproduction to the favourable periods are more likely to be successful than those that reproduce during unfavourable periods. Climatic variables that have a seasonal nature impose seasonality, to some extent, on dietary variables. This has effectively shaped the evolution of reproductive systems such that the degree to which births are restricted to a discrete period of

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the year often reflects the degree to which the habitat is seasonal. Sadlier (1969) identified three basic patterns of reproduction in ungulates. First, continuous reproduction is often observed in tropical and equatorial regions where environmental conditions are favourable for most of the year. Second, unpredictable reproduction characterises opportunistic species that inhabit environments in which the optimal season for reproduction is short and can occur at any time of the year. Third, regular, predictable reproduction is the rule in temperate regions.

Seasonal breeding

Many species restrict their reproductive activities to a "breeding season" such that environmental conditions are optimal for the survival of neonates (Macarthur & Lewis 1967). The resulting pronounced clustering of reproduction is a fairly widespread phenomenon in the animal kingdom (Janzen 1971, Ims 1990). Restricted breeding is the result of interactions between organisms and their physical environment and other species (Ims 1990). Restricted breeding requires a species to be able to time reproduction so that birth occurs at the most propitious time.

There are two main classes of cue that organisms can use to time reproduction, namely internal and environmental cues. These are not mutually exclusive. Internal cues arise from an endogenous rhythm in the organisms themselves and social cues constitute signals passed between individuals within a population. For example, oestrus can be synchronised among a group of female ungulates by the introduction of a male (Marais & Skinner 1993). Environmental cues are directly connected to climatic seasonality, such as photoperiod, temperature, humidity and food availability. As photoperiod is the most constant of the potential cues, it is not surprising that it is used by many classes of animal as a means of cueing their reproductive activities.

Although the Dutch manipulated light to get birds to sing out of season as early as the seventeenth century (Follet 1985), the use of photoperiod as a predictive cue was first demonstrated in a mammal by Baker & Ransom (1932). They observed that in a colony of field voles (*Microtus agrestis*) held on 15 hours of daylight, reproduction was normal. However, when

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exposed to nine hours of light a day, reproduction was blocked. Since then, photoperiodism has been shown to influence reproduction in most mammalian orders (Arendt 1995), but has been most thoroughly studied in hamsters and sheep.

The photoperiodic system

Photoperiodism requires three components. First, a photoreceptor is needed to detect light and a neural system is needed to monitor fluctuations in day-length to enable a distinction between long and short days. Second, there must be a neural pathway linking the neural system to the neuroendocrine apparatus. Third, there is the endocrine system, comprising the pituitary gonadotrophins, gonads and gonadal steroids.

The photoneuroendocrine pathway is summarised in Figure 2. Mammals use their eyes as photoreceptors for the measurement of day length. Photoperiodic information flows from the retina along the monosynaptic retinohypothalamic tract, a system of neurones separate from the visual system, which terminates in the suprachiasmatic nuclei just above the optic chiasma. After information has passed through the suprachiasmatic nuclei, neural impulses pass through the paraventricular nucleus of the hypothalamus and down the intermediolateral cell column of the spinal cord. Preganglionic sympathetic nerves leave the thoracic part of the cord and ascend the neck to synapse in the superior cervical ganglia. From here, postganglionic sympathetic neurones innervate numerous structures in the brain, including the pineal gland.

The pineal gland is one of several structures innervated by neural projections from the superior cervical ganglia and is central to modulation of reproduction in mammals. Thus, pinealectomised animals fail to respond appropriately to photoperiodic information (Barrell & Lapwood 1979, Cassone 1992). The most important hormone secreted by the pineal gland is the indoleamine, melatonin (Lerner *et al.* 1958). Melatonin is also produced by a number of anatomical structures other than the pineal gland (e.g. the Harderian gland and digestive tract, Bubenik *et al.* 1976*a*, *b*). However, the fact that pinealectomised animals have virtually no melatonin present in their plasma (Bittman *et al.* 1983, Wayne *et al.* 1988) highlights the importance of this organ in producing a melatonin signal. The pineal modulates physiological

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thereafter to a depth calculated to correspond to the desired position of the tip within the third ventricle.

The final cannula position was confirmed by frontal and lateral X-rays (Figure 4) before being fixed to the skull with dental acrylic (Figure 5). The cannula was capped with a plastic stopper to prevent CSF leakage and maintain sterility. Finally, the cannula was protected by a cylindrical Teflon cap (OD: 30 mm; ID: 22 mm; height: 22 mm), fixed to the skull with four stainless steel screws and dental acrylic.

Calculation of the time delay (lag time) when collecting CSF

The total volume of the collection apparatus through with CSF flowed between the ventricle and collection tube was calculated as the sum of the volume of the catheter and the line connecting the catheter to the collection tube. The total volume was 167.21 μl (Box 1). The time delay (in seconds), calculated as the product of the dead volume of the apparatus and the flow rate (Box 2), was calculated separately for each animal after each sampling occasion. The lag time calculated for each sampling session was used to ascertain the actual time at which a CSF sample was collected. This allowed the position of the hormone data points to be correctly plotted with respect to time.

Box 1. Calculation of the volume of dead space in the collection apparatus.

v_1 = catheter volume	v_2 = line volume
r_1 = catheter diameter	r_2 = line diameter
h_1 = catheter length	h_2 = line length
$v_1 = \pi r_1^2 \times h_1$	$v_2 = \pi r_2^2 \times h_2$
$= \pi (0.298\ mm)^2 (430\ mm)$	$= \pi (0.063\ mm)^2 (3850\ mm)$
$= 119.56\ mm^3$	$= 47.25\ mm^3$
If $1\ mm^3 = 1\ \mu l$ then	If v = total dead volume then
$v_1 = 119.96\ \mu l$	$v = v_1 + v_2$
$v_2 = 47.25\ \mu l$	$= (119.96 + 47.25)\ \mu l$
	$= 167.21\ \mu l$

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Box 2. Calculation of the flow rate and correction factor.	
Flow rate (f) of CSF = volume of CSF flowing per time unit.	d = delay v = total dead volume f = flow rate of CSF
Eg. If 30 μl flows in 60 s, then $f = (30 \div 60)\ \mu l/s = 1.25\ \mu l/s$	$d = f \times v$ $= f \times 167.21\ \mu l$ $= (f \times 167.21)$ s
Thus, f for each animal is multiplied by 167 to give the delay in s.	

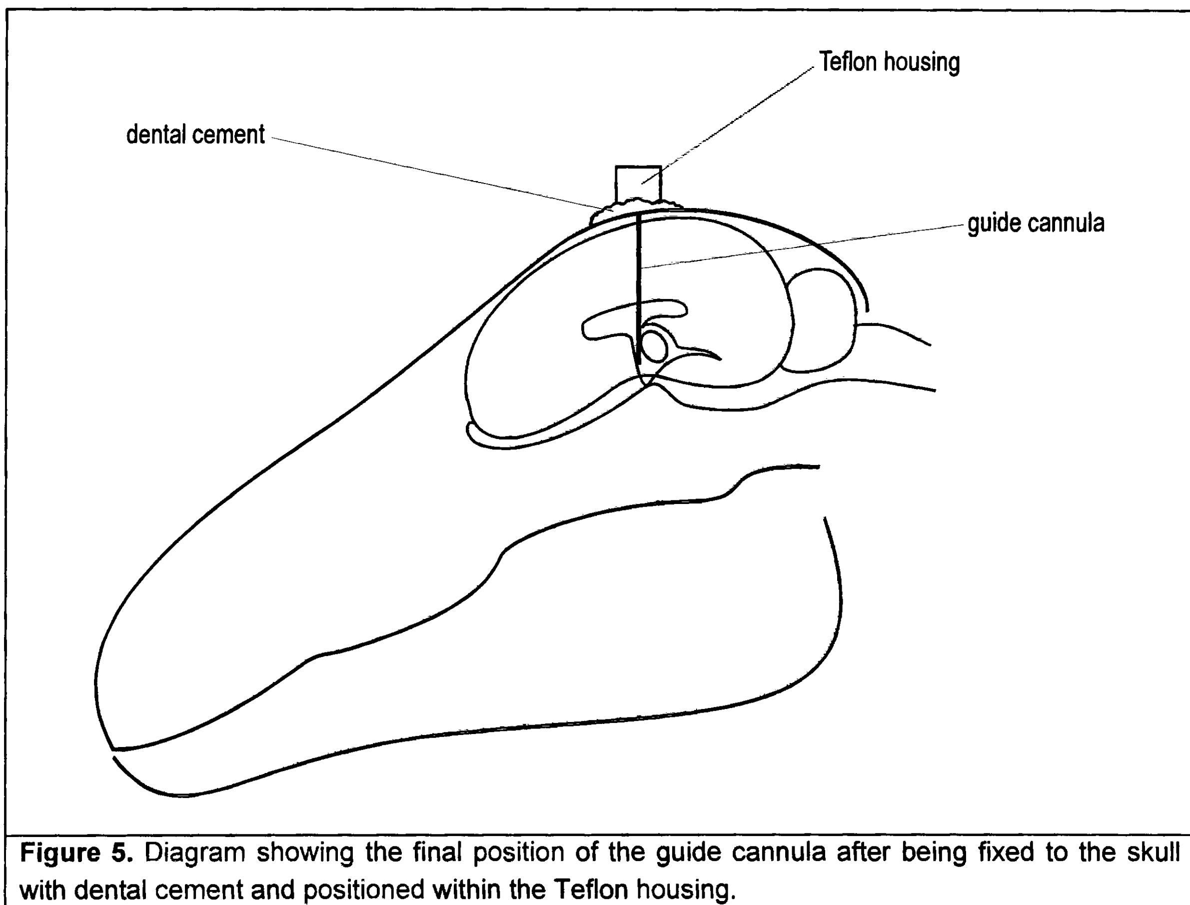

Figure 5. Diagram showing the final position of the guide cannula after being fixed to the skull with dental cement and positioned within the Teflon housing.

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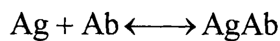
Radioimmunoassay

As radioimmunoassays formed a major technical component of my thesis, it is appropriate that a detailed discussion of this concept is included. I have only included the details of the assays that I conducted, namely the progesterone and LHRH radioimmunoassays. However, the radioimmunoassay for LH is based on precisely the same basic principles and the assay protocols for LH, LHRH and progesterone are very similar.

The radioimmunoassay has revolutionised endocrinology because it is more sensitive, specific and practicable than earlier techniques such as the biological assay. The thermodynamic reaction that forms the basis of the radioimmunoassay is presented below.



$$Ag + Ab \longleftrightarrow AgAb$$

A ligand (antigen, Ag), and a binder (antibody, Ab), combine to form an antigen-antibody (AgAb) complex. The binding reaction is reversible. The rate of dissociation is considerably slower than the association rate and the observed amount of AbAg complex is the result of the combined rates of association and dissociation. If the reaction proceeds until it reaches equilibrium, at which point the total amount of Ag, Ab and AgAb are constant. The exact concentration of each molecule at equilibrium depends on the initial amounts of the starting reagents. However, at equilibrium, the ratio of the products of the concentrations on each side of the equation is equal to a constant, K, called the affinity constant. Thus, given an unvarying quantity of Ab of fixed K value, the ratio of bound to free Ag at equilibrium is quantitatively related to the total amount of Ag present. This is the basic principle of all binding assays.

Requirements for a radioimmunoassay

Highly purified Ag is essential for a radioimmunoassay. While some Ags (e.g. large proteins) produced from natural sources present problems due their relative impurity (see Hwang *et al.* 1971), the steroids used in the radioimmunoassays in this study are prepared synthetically and are highly purified. Labeled Ag or tracer is used to determine the distribution of unlabelled Ag between bound and free phases. Tracers should react with Ab in as similar a manner as

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unlabelled Ag as possible. Antibodies should be specific to the Ag for which they are intended to be used. The Ab used in the progesterone assay was supplied by R. P. Millar (Dept Chemical Pathology, University of Cape Town). The progesterone Ab (#1529) was raised in goats to progesterone-11-succinyll-bovine serum albumin. Significant cross-reactivity occurs with 11α-hydroxyprogesterone (85%), 17α-hydroxyprogesterone (12.5%) and 5β-pregnane-3,20-dione (12.5%).

Separation of bound and free antigen

Fractional precipitation is widely used and its simplicity makes it highly practical. The principle is simple: separation material (salts or organic solvents) is added after the AgAb reaction is complete. The concentration is high enough so that the antibody and AgAb complex become insoluble and precipitate, while the unbound Ag remains in solution. The precipitate is packed by centrifugation and radioactivity is determined in either the pellet (bound fraction) or supernatant (free fraction). This was the method used in the LHRH assay protocol, the organic solvent being methanol.

Steroid hormones usually circulate in serum bound to proteins that have affinities similar to those of antibodies. The most common method of separating Ab-bound and free steroid hormones is with dextran-coated charcoal. Basically, the surface of small molecules of charcoal ($< 60\ \mu m$) are coated smaller dextran molecules, essentially producing a microscopic "sieve". When a suspension of this material is added to the assay tube, the smaller unbound steroid molecules are adsorbed to the charcoal and the larger Ab and AgAb molecules left in solution. The charcoal and adsorbed Ag is then compacted by centrifugation and radioactivity of the steroid-Ab complex determined in the supernatant. This was the method followed in the progesterone radioimmunoassay.

Extraction of antigen from biological fluids

Extraction for purification of the Ag is usually carried out because the Ag is closely related to substances that can react equally well in the assay, or the Ag is associated with other materials

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in the biological fluid, such as binding proteins, which prevent its participation in the assay reaction. In the case of steroid hormones, naturally occurring Abs are often used that have a broad specificity and cross-react with molecules other than the Ag.

If the Ag is to be purified, an immiscible organic solvent is used which extracts the Ag. The latter method was employed in the progesterone assay to extract progesterone from plasma. Specifically, excess petroleum ether (an organic solvent that selectively takes up progesterone) was added to each sample tube, the tubes shaken and then frozen. The unfrozen fraction, which contains the extracted progesterone, was then decanted, the ether evaporated and the remains reconstituted in buffer. A similar approach was followed in the LHRH assay, except that methanol was used to extract the LHRH.

Calculation of results

A standard curve is the basic requirement for quantification of the Ag concentration in unknown samples. A standard curve is created by incubating fixed concentrations of tracer (labeled) Ag and Ab with different concentrations of unlabelled Ag. If the percentage of tracer bound is plotted on a logarithmic scale against serial dilutions of the Ag, the result is a sigmoid curve (Figure 6). At the upper end of this curve, small changes in the amount of unlabelled Ag produce minor changes in the distribution of the bound and free phases. Similarly, at the lower end, the majority of Ag is free. However, between these two extremes, relatively small changes in the amount of Ag cause a significant alteration in the distribution of bound and free Ag. This steep part of the slope thus represents the effective range of an assay. When a sample of unknown Ag concentration is substituted for the standard and the same quantity of Ab and tracer is used, the value determined for the distribution of bound and free phases will be equivalent to some value on the horizontal scale of the standard curve. This value is then corrected for prior extraction.

Although the convention used in our laboratory is a semi-logarithmic plot of percentage tracer bound against the log of concentration of unlabelled Ag, there are many alternative methods of presenting the same data. Each method has advantages and disadvantages, all of which are

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reviewed by Chard (1981). The "four-parameter logistic" curve is popular because it satisfies the requirement for wide use in routine assays, namely (*i*) very few (preferably only four) parameters, (*ii*) flexibility of position, slope and shape, (*iii*) monotonic form (no reversals of slope) and resistance to outliers (Dudley *et al.* 1985) and (*iv*) it closely approximates the mass-action equation (Finney 1983). The general form of equation is:

$$\text{Expected response at dose} = d + (a - d)/[1 + (\text{dose}/c)^b]$$

where a is the expected response at zero dose of analyte; b is the slope factor or exponent; c is the concentration of analyte with an expected response exactly halfway between a and d; and d is the expected response for infinite analyte concentration. The response curve is fitted to raw counts by the method of iteratively re-weighted non-linear least squares.

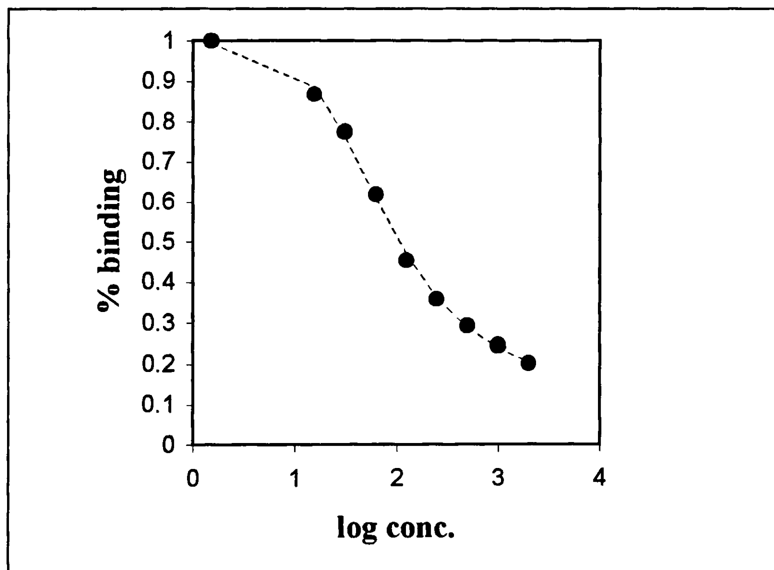

Figure 6. Example of the sigmoid "standard" curve. Each point (closed circles) represents the response of a standard preparation. The broken line represents the "best fit" of the dose response curve, where the expected response at dose = $d + (a - d)/[1 + (\text{dose}/c)^b]$, using an iteratively re-weighted non-linear least squares method to fit the curve.

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Precision

Precision is defined as the extent to which a given number of same samples agree with their mean, i.e. the amount of variation in the estimation (Midgley *et al.* 1969). In general, plasma or hormone pools are replicated in each radioimmunoassay to provide an estimate of both the within- and between-assay variation. Thus, to determine the degree of variation within an assay, a series of samples of a quality control pool are treated identically to the unknown samples. These quality control samples are placed at the beginning, middle and end of the tube series, or spaced at regular intervals throughout the tube series, to take account of any drift in the assay. The mean and standard deviation of all the quality control samples is then calculated and the result expressed as a coefficient of variation (CV, standard deviation as a percentage of the mean). The same quality control samples are used to calculate between-assay variation. Here, each set of quality control samples is treated as a single sample and a CV calculated.

Confidence limits

Confidence limits are normally the simple application of a CV to a range of values, e.g. if a CV is 5 % then the 95 % limits of an estimate would be the value plus and minus 10 %. However, in the radioimmunoassay, the precision of estimates varies according to the dose. Thus, the confidence limits are calculated using weighted values to take into account this variation.

Sensitivity

Sensitivity is normally defined as the minimal detection limit of an assay. This, in practical terms, refers to the least concentration of unlabelled Ag that can be distinguished from a sample containing no unlabelled Ag, considering the confidence limits of each point. Thus, the minimal detection limit can vary between assays.

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Specificity

The degree to which an assay responds to substances other than that for which the assay was designed determines its specificity. Since there is extensive structural homology between various steroids, it is not always possible to generate sufficiently specific antisera to measure the hormones without initial extraction and separation techniques.

Assay validation

To determine the doses of unknown samples both the reference preparation and hormone must interact similarly with the Ab and exhibit parallel dose-response curves. Thus, when a sample is included at two or more dilutions, determining whether the concentrations obtained at different parts of the response curve are consistent is a generalised test of "parallelism". If the concentrations are the same, the dilution curve should parallel the dose-response curve. If both curves are plotted on the same semi-logarithmic axes, their slopes should be the same (Figure 7).

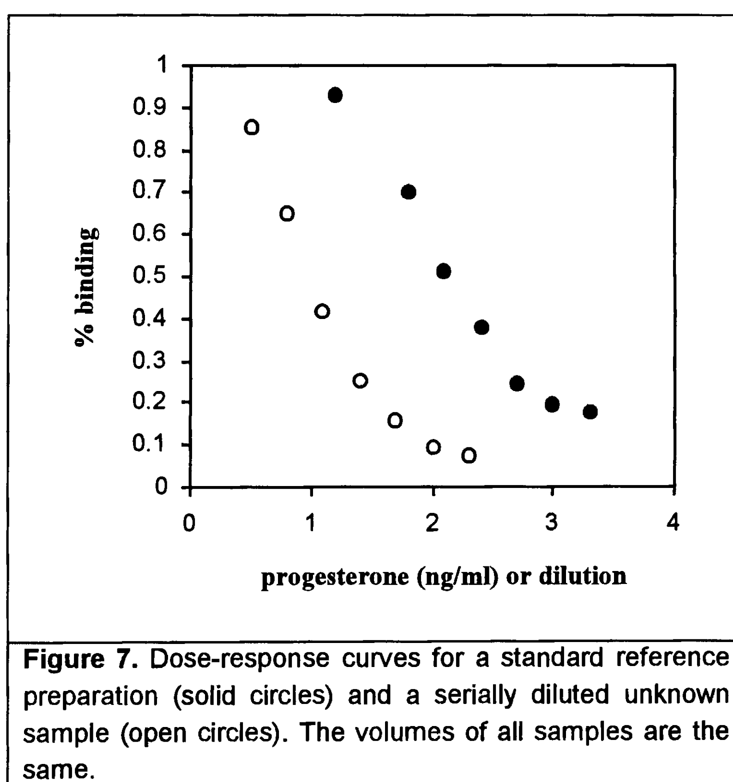

Figure 7. Dose-response curves for a standard reference preparation (solid circles) and a serially diluted unknown sample (open circles). The volumes of all samples are the same.

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A further means of validating the assay technique is determining whether the biological fluid (plasma in the case of the progesterone assay, and CSF in the case of the LHRH assay) has any discernible effect on the response obtained. Thus, standards of known concentration are added to stripped plasma (plasma from which all accessible steroid hormones have been extracted). If the plasma has no differential effect on the response obtained at different concentrations, the observed and expected responses should be the same. A simple means of visualising this is by using a bivariate plot of observed *vs* expected concentration (Figure 8). However, quantifying the amount of deviation of sample dilutions from their expected values forms the basis of testing for statistically significant differences between the dose-response and dilution curve. This usually takes the form of a chi-square test (details are presented in Appendix 3 of Dudley *et al.* 1985).

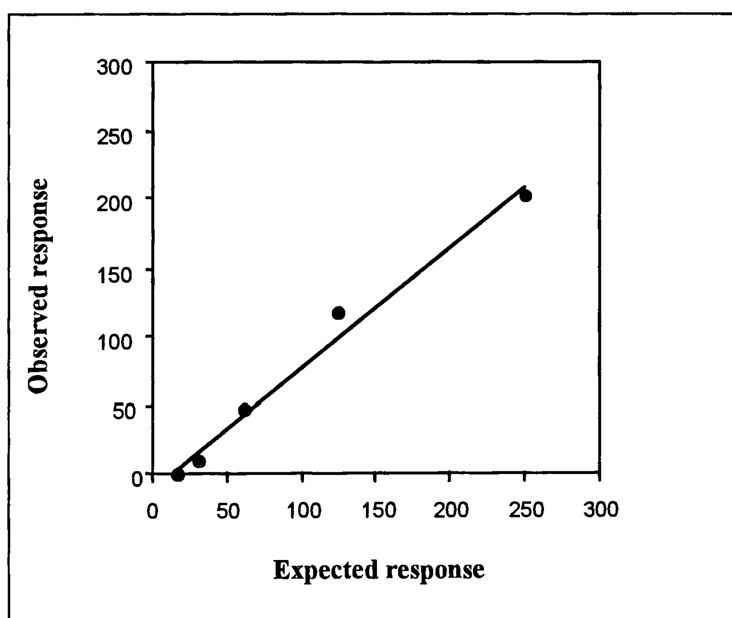

Figure 8. Bivariate plot of the expected and observed concentration (response) of progesterone in stripped plasma samples "spiked" with progesterone. Concentrations are in pg progesterone per ml sample. For the relation depicted by the solid line, $y = 0.88x - 10.21$ and $r^2 = 0.99$. The observed and expected responses were not significantly different ($p > 0.05$, chisq. < 0.003, $df = 4$).

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Progesterone radioimmunoassay

1. *Extraction*

Progesterone was extracted from plasma samples by adding excess (4 ml) diethyl ether to each tube containing plasma. The samples were shaken for 10 min and frozen for 1 h (– 70°C). The unfrozen ether phase was then decanted, the ether evaporated under a stream of nitrogen gas and the remainder reconstituted in buffer (PBS, pH 7 – 7.5). The mean recovery of progesterone for 12 assays was 80 ± 1 %.

2. *Assay protocol*

A range of standards was prepared with concentrations of 0, 7.8, 15.6, 31.2, 62.5, 125, 250, 500, 1000 and 2000 pg/ml unlabelled progesterone (Sigma No. P-0130), respectively. Each standard was assayed in triplicate. Ab was added to each standard and sample tube and the tubes shaken. All tubes were then incubated for 10 min at 25°C. Tritiated progesterone (*c.* 20 000 dpm/tube) was then added. Tubes were shaken again and held at 4°C for between 12 and 18 h. Unbound steroid was exposed to dextran (T70, Pharmacia, Uppsala)-coated charcoal (Merck, Darmstadt, Germany) for 12 min and then the bound and unbound phases separated by centrifuging at 2 500 rpm for 15 min. The supernatant was decanted into 8 ml hinge-cap vials (Packard Instruments, Cape Town, South Africa) and 4 ml of scintillation cocktail (Ultima Gold XR, Packard Instruments) added. After 3 h, radioactivity was determined in a Packard 1 500 liquid scintillation counter. The protocol is summarised in Table 1.

3. *Assay validation*

First, the recovery of exogenous progesterone from "stripped" plasma (plasma from which steroid hormones have been removed with dextran-coated charcoal) was 77.5 %. Recovery was independent of hormone concentration (chi-square test of observed *vs* expected response, chisq. < 0.003, $p > 0.05$, $df = 4$), demonstrating that neither of these parameters significantly affect the response.

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Second, the slope of logit-log transformed curves of the standards and serial dilutions of an unknown plasma sample were not significantly different, suggesting that sample volume does not have any appreciable effect on the response.

LHRH radioimmunoassay

1. *Extraction*

An excess volume of methanol (2 ml) was used to extract the LHRH from CSF samples. After the methanol was added, tubes were shaken and held at – 20°C for at least 30 min. After centrifuging for 30 min (3 200 rpm), the supernatant containing the LHRH was decanted into glass tubes. The methanol was evaporated overnight (Savant, Faringdale, USA) after which each sample was reconstituted in buffer (PBS). The mean recovery using this method was typically > 80 %.

2. *Assay protocol*

A range of standards was prepared with concentrations of 0, 0.35, 0.7, 1.5, 3.1, 6.3, 12.5, 25, 50, 100 and 200 pg/ml unlabelled LHRH, respectively. Each standard was assayed in triplicate; unknown samples could only be assayed once. Ab (BDS 037) was added to each standard and sample tube and the tubes shaken. All tubes were then incubated overnight (*c.* 12 h) at 4°C. Tritiated LHRH was added (*c.* 20 000 dpm/tube) and the tubes held overnight at 4°C. The bound LHRH (LHRH-Ab complex) and unbound Ab were precipitated by adding 2 ml ethanol (at 4°C) to each tube. After 30 min at 4°C, the tubes were centrifuged. After this, the supernatant was discarded and radioactivity in the pellet was determined after 30 min using a crystal multi-detector RIA system (Packard Instruments). The assay protocol is summarised in Table 2.

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3. *Assay validation*

The LHRH radioimmunoassay using the same reagents as were used in this study has been previously validated for Ile-de-France ewes by Caraty *et al* (1980). It was thus unnecessary to repeat this in the present study.

Table 1. Summary of the radioimmunoassay protocol used in the progesterone assay. [1]Any unknown, extracted plasma sample. [2]Quality control plasma pool.

Tube	Description	^{3}H-P	Ab	PBS	Sample
1-3	Non-specific binding	100 μl	none	200 μl	none
4-6	Total counts	100 μl	none	900 μl	none
7-9	Zero standard	100 μl	100 μl	none	standard
10-36	Standards 1-9	100 μl	100 μl	none	standard
37-38	Quality control 1	100 μl	100 μl	none	pool
39-105	Plasma samples	100 μl	100 μl	none	unknown[1]
106-107	Quality control 2	100 μl	100 μl	none	pool[2]
108-176	Plasma samples	100 μl	100 μl	none	unknown
177-178	Quality control 3	100 μl	100 μl	none	pool
179-181	Recovery estimate	100 μl	none	none	unknown
182-184	Extraction control	100 μl	100 μl	none	none

Table 2. Summary of the radioimmunoassay protocol used in the LHRH assay.

Tube	Description	^{3}H-P	Ab	PBS	Sample
1-3	Non-specific binding	100 μl	none	200 μl	none
4-6	Total counts	100 μl	none	none	none
7-9	Zero standard	100 μl	100 μl	none	standard
10-29	Standard 1-10	100 μl	100 μl	none	standard
30-	Plasma samples	100 μl	100 μl	none	unknown

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Chapter 3

Do springbok cue reproduction according to the environment?

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Introduction

Springbok are generally regarded as aseasonal breeders (Skinner & Louw 1996). In their natural habitat, springbok breed throughout the year, although there are definite "pulses" in the annual frequency distribution of lambing (Skinner *et al.* 1974; Jackson 1995). The relatively unrestricted lambing pattern of springbok implies that springbok are incapable of restricting lambing to a greater degree than is that observed. This hypothesis implies that springbok are unable to cue reproduction by means of environmental *zeitgebers* such as photoperiod. Alternatively, springbok are able to further restrict lambing, but fail to do so. In contrast to the former explanation, this hypothesis does not preclude the existence of photoperiodism. These two hypotheses are mutually exclusive.

Photoperiod is the most ubiquitous environmental *zeitgeber* used by mammals to time reproduction in non-equatorial regions. The reason is that changes in the photoperiod are highly regular and stable. Animals use changes in day length to deduce the prevailing season and behave accordingly. For instance, sheep respond to increasing day length between the autumnal equinox and summer solstice and mate in autumn (Malpaux *et al.* 1989), in anticipation of propitious summer conditions when they lamb. The magnitude of difference between summer and winter day lengths increases with increasing latitude and hence the degree to which births are restricted increases with increasing latitude (Baker 1938).

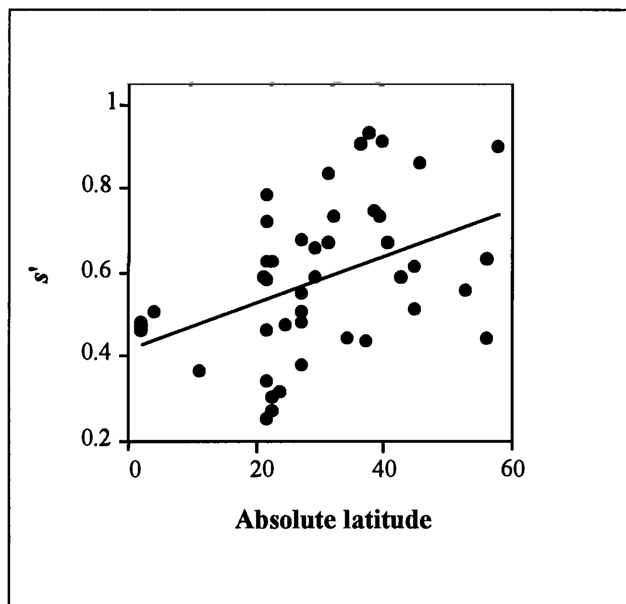

Figure 9. The relationship between latitude (absolute latitudinal position north or south) and the degree of restriction in 45 ungulate species maintained in the same captive environmental conditions in London Zoological Gardens. The solid line depicts the regression $y = 0.006x + 0.25$ ($r^{2f} = 0.19$, $p < 0.05$, $n = 45$). Data are from Zuckerman (1953) and are presented fully in Table 4.

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Highly restricted breeders generally limit lambing to that period of the year during which environmental conditions are most favourable. When the duration of this period is short (in high latitudes, for instance), breeding is highly restricted. However, if highly restricted breeders are moved to an environment in which the favourable period is longer, the length of the calving period increases accordingly and restriction decreases (Figure 9). For example, sheep moved from Europe, where they are restricted breeders, to the equator are unrestricted and capable of breeding throughout the year (Sadlier 1969). Thus, to determine whether springbok are able to use photoperiod to cue reproduction, we can examine whether they alter the degree of lambing restriction in different environments. If springbok do not use photoperiod to cue reproduction, the degree to which springbok births are restricted should not correlate with latitude (an index of photoperiod). Alternatively, if springbok use photoperiod to cue reproduction restriction should be positively correlated with latitude.

Methods

Data were assembled from two sources to address this question, namely populations of springbok translocated from the Southern to the Northern Hemisphere and populations from different localities within the southern African subregion. The former data allow an assessment of restriction according to latitudinal differences, in particular in response to a shorter favourable environmental period; the latter data are for populations from different longitudes, which represent a gradient of environmental seasonality. Details appear in the legend of Figure 10.

For each geographic locality, the monthly mean of the number of births, rainfall and temperature was calculated and converted to a relative frequency distribution. The degree to which lambing was restricted (s') was then calculated for each population (see Appendix 2, p. 90, for details). If s' has a value close to 1 (e.g. 0.9), this is considered highly restricted. Conversely, restriction is low if s' is close to zero. The degree of variability of rainfall and temperature was calculated in a similar manner using the variance of a frequency distribution, with the frequency interval being 12 months.

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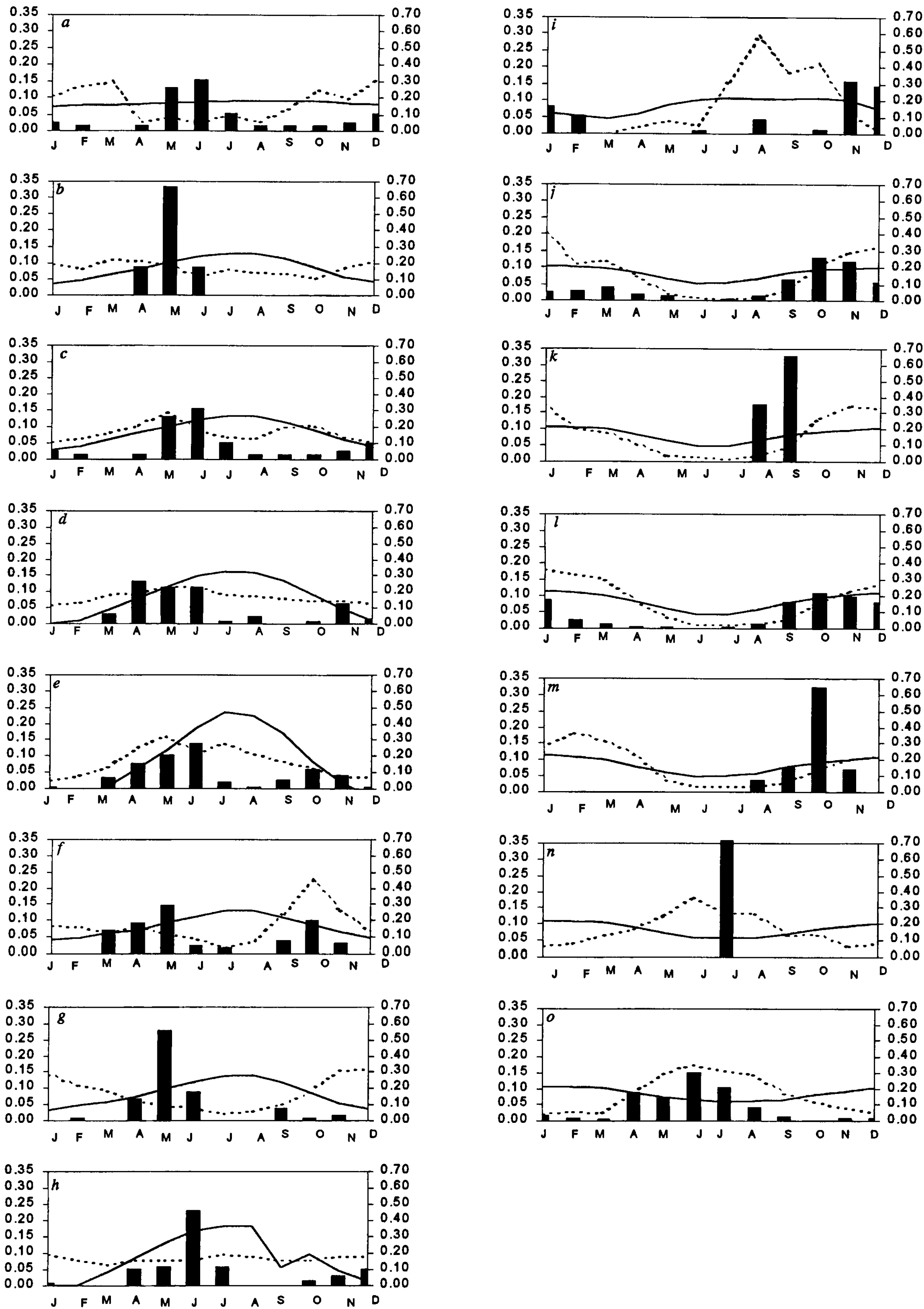

Figure 10. Calving in several springbok populations. Solid lines, temperature and broken lines, rainfall (both 1st *y*-axis). Bars (2nd *y*-axis) represent the relative proportion of births. *a*, Honolulu Zoo, Hawaii, USA (21°19'N 157°50'W); *b*, Jackson Zoo, Mississippi, USA (32°20'N 90°11'W); *c*, Fort Worth Zoo, Texas, USA (32°45'N 97°20'W); *d*, Saint Louis Zoo, Missouri, USA (38°40'N 90°15'W); *e*, Denver Zoo, Colorado, USA (39°45'N 105°00'W); *f*, Réserve Africaine de Sigean, Narbonne, France (43°11'N 3°0'E); *g*, Woodland Park, Washington, USA (47°45'N 120°30'W); *h*, Tierpark Carl Hagenbeck, Hamburg, Germany (53°32'N 9°59'E); *i*, Kalahari-Gemsbok National Park, southern Africa (25°15'S 20°30'E); *j*, Pretoria Zoo, Pretoria, South Africa (25°45'S 28°10'E); *k*, Athole Research Station, Ermelo, South Africa (26°31'S 29°59'E); *l*, Lombard Reserve, Bloemhof, South Africa (27°38'S 25°37'E); *m*, Benfontein, Kimberley, South Africa (28°45'S 24°45'E); *n*, Springbok, South Africa (29°40'S 17°53'E); *o*, Tygerberg Zoo, Cape Town, South Africa (33°52'S 18°43'E).

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An appraisal of the two hypotheses presented in the Introduction requires an assessment of the strength of the correlation between restriction in calving and variability in environmental parameters. Thus, the relationship between lambing restriction and latitude (an index of photoperiod) was assessed, as well as the relationship between lambing restriction and variability in rainfall and temperature. This was done for both captive and wild springbok populations.

Results

Lambing in the natural habitat

Wild springbok populations in their natural habitat are moderately restricted (Figure 10). The mean degree of restriction for wild springbok populations in southern Africa is 0.60 ± 0.28. Typically, lambing occurs throughout the year in wild populations, although there are definite "pulses" in lambing distributions (Figure 10). In the summer rainfall region of South Africa, the pulses occur from September to January, which is when rainfall is most likely to occur. Data for the wild population in the winter rainfall region of South Africa has a distinct birth pulse in June/July, which is when rainfall is most likely to occur in this region.

Restriction according to latitudinal differences

Springbok translocated to the Northern Hemisphere tend to alter their lambing pattern so that the birth pulses coincide with spring/summer in the months of May/June, rather than in September/October as in the Southern Hemisphere. This was true for all populations for which data were available (Figure 10). The degree of restriction in Northern Hemisphere zoo populations was 0.41 ± 0.02 and 0.49 ± 0.03 for zoo populations in the Southern Hemisphere. These values are not significantly different (Student's t-test, $p > 0.05$, $n = 9$).

The degree of restriction in the Northern Hemisphere zoos varied from 0.27 (Beekse Bergen, Netherlands) to 0.67 (Jackson zoo, USA). When each locality was analysed for correlations between rainfall and births, temperature and births, and latitude and births, respectively, no

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clear trend emerged (Table 3). Rainfall and births were significantly positively correlated in only two of ten localities, and temperature and births significantly correlated in just one locality.

Restriction according to differences in environmental seasonality

Wild springbok populations from various localities in southern Africa showed a slight trend toward increasing restriction as longitude increased, although this was not significant (Figure 11); however, the correlation between restriction and rainfall, and restriction and temperature, was not significant for either wild or captive populations (Table 3).

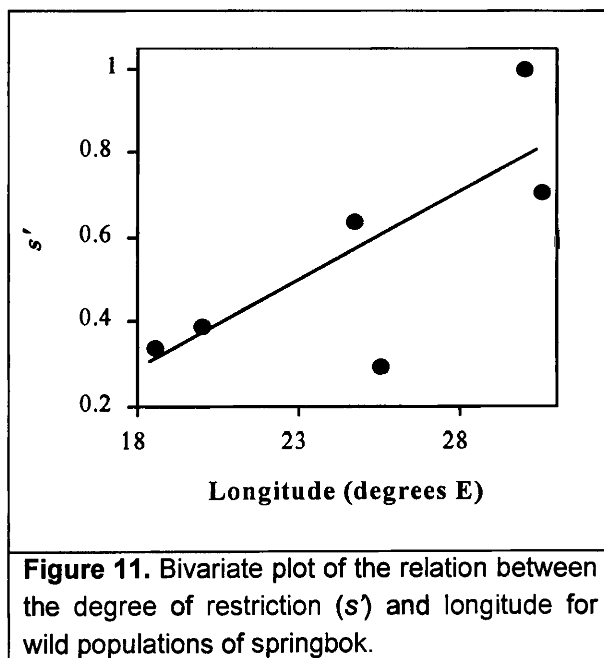

Figure 11. Bivariate plot of the relation between the degree of restriction (*s'*) and longitude for wild populations of springbok.

Discussion

Springbok are moderately restricted in their natural habitat but seem unable to increase restriction significantly in response to an increasingly seasonal environment. Although the southern African localities used in my analysis represent a gradient in environmental

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seasonality, this gradient may not be steep enough to induce significant alteration in restriction. Springbok translocated to the Northern Hemisphere do switch their lambing pattern so that

Table 3. Details for regression analyses of the degree of restriction (s') and latitude/longitude, temperature and rainfall for wild and captive springbok populations.

Correlation variables		POPULATION TYPE	
		Captive	Wild
Restriction *vs* Latitude	eqn	$s' = -0.002x + 0.34$	—
	r^2	0.03	—
	p	0.71	—
Restriction *vs* Longitude	eqn	—	$s' = 0.04x - 0.48$
	r^2	—	0.58
	p	—	0.08
Restriction *vs* Temperature	eqn	$s' = -1.36x + 0.50$	$s' = -0.001x + 0.001$
	r^2	0.09	0.00
	p	0.51	0.92
Restriction *vs* Rainfall	eqn	$s' = -1.68x + 0.48$	$s' = -0.167x + 0.002$
	r^2	0.04	0.78
	p	0.65	0.12

birth pulses coincide with spring/summer, but there is no evidence that this switch is due to photoperiodism. Furthermore, although the pattern is switched, the degree of restriction in southern and Northern Hemisphere populations is similar. It thus seems that, while springbok lambing patterns generally reflect seasonality in environmental conditions, the degree to which lambing is restricted is relatively stable. The most likely explanation for this observation is that phylogeny might constrain restriction plasticity. Evidence in support of this is presented in Appendix 2.

There are obvious birth pulses in nearly all springbok populations. Birth pulses could be the result of the use of photoperiod and/or rainfall as a cue. However, birth pulses are a inherent characteristic of populations and can result from differential seasonal infant mortality and incidental timing as a result of the reproductive cycle, both of which are essentially

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environmental factors, without the existence of adaptations to cue reproduction (see Appendix 3, p. 97). Indeed, there is no clear relationship between rainfall or temperature and restriction. Furthermore, there is no doubt that survival in the wild is lower in dryer years (Penzhorn 1975, Bigalke *et al.* 1975) and Skinner & Van Zyl (1970*b*) suggested that culling and exceptional rainfall may advance oestrus, inducing some ewes to lamb twice in one year. These facts, together with Jackson's (1995) observation that springbok lambing in the southern Kalahari is synchronous, but that lambing periods are not coincident in consecutive years, tend to corroborate the assertion that pulses in springbok populations are most likely environmentally induced rather than directly adaptive. The model presented in Appendix 3 supports this contention.

In conclusion., the evidence presented in this Chapter strongly suggests that springbok do not use photoperiod or other climatic *zeitgebers* to cue reproduction. However, it is impossible to assess the degree to which springbok innately restrict reproduction without controlling for the effect(s) of other factors, such as nutrition and predation. Certainly, predation pressure can often determine the degree of restriction in wild populations of ungulates (Rutberg 1987) and phylogenetic constraints may also play a role (Appendix 2). Thus, if these factors can be controlled or—ideally—eliminated, it should be possible to assess the innate degree of restriction. This forms the subject of Chapter 4.

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Table 4. Summary of the data used in Figure 9. Birth record data were initially converted into relative annual frequency distributions and subsequently the degree of restriction was calculated for each species as defined in Appendix 2. Degrees north of the equator are positive and degrees south, negative.

Species	Common name	Approximate latitude	Degree of restriction (s')
Ammotragus lervia	Barbary wild sheep	20	0.42
Anoa depressicornis	Anoa	3	0.34
Axis axis	Axis deer	20	0.07
Bibos frontalis	Gayal	25	0.34
Bison bison	American bison	40	0.43
Bos grunniens	Yak	32	0.27
Bos indicus	Zebu	21	0.12
Bos taurus	English wild cattle	53	0.27
Bubalus bubalis	Indian (Water) buffalo	20	0.47
Capra caucasia	Caucasian tur	43	0.71
Capra falconeri	Markhor	30	0.58
Capra hircus	Domestic goat	37	0.54
Capra hircus	Domestic goat	53	0.77
Capra hircus cretensis	Cretan ibex	35	0.79
Capra sibirica	Himalayan ibex	29	0.68
Cephalophus maxwelli	Maxwell's duiker	– 1	0.29
Cervus canadensis	Wapiti deer	42	0.35
Cervus elaphus	Red deer	50	0.40
Cervus hanglu	Cashmere deer	34	0.76
Connochaetes taurinus	Blue wildebeest	– 25	0.39
Dama dama	Fallow deer	36	0.59
Gazella dorcas	Dorcas gazelle	27	0.42
Gazella subgutturosa	Persian gazelle	25	0.53
Hemitragus jemlahicus	Thar	29	0.51
Hybrids of above two		23	0.31
Hyelaphus porcinus	Hog-deer	22	0.14
Kobus leche	Lechwe waterbuck	– 20	0.29
Muntiacus reevesi	Reeves' muntjac	35	0.27
Odocoileus hemionus	Mule-deer	38	0.52
Odocoileus mexicanus	Mexican deer	19	0.43
Odocoileus virginianus	American fallow deer	37	0.58
Oryx algazel	Sabre-horned oryx	20	0.29
Ovis aries	Soay sheep	20	0.57
Ovis burrhel	Burrhel wild sheep	27	0.50
Ovis musimon	Mouflon	42	0.45
Ovis vignei	Punjab wild sheep	21	0.47
Rangifer tarandus	Caribou	55	0.76
Rucervus duvaucelii	Swamp-deer	20	0.63
Rusa moluccensis	Molucca deer	– 1	0.31
Rusa unicolor	Sambur deer	10	0.19
Syncerus cafer	Cape buffalo	– 25	0.31
Tragelaphus oryx	Eland	– 25	0.20
Tragelaphus scriptus	Harnessed antelope	– 1	0.31

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C h a p t e r 4

Is the springbok seasonal?

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Introduction

There is little evidence from population studies that springbok use environmental cues such as temperature, rainfall and photoperiod to cue reproduction (see Chapter 3, p. 31). While the results of analyses based on such indirect evidence are instructive, the fact that the data used were derived from populations that were not subject to any formal control means that the existence of photoperiodism cannot be discounted. Therefore, to establish whether springbok are seasonal breeders, the possible effects of confounding factors, such as the influence of nutrition, need to be accounted for.

Ungulates that are photoperiodic generally have the following characteristics. They have a discrete (often short) breeding season or reproductive period; females usually come into oestrus at about the same time; the onset of oestrus usually occurs at a similar time every year and animals that mature before this date usually delay sexual behaviour until the appropriate time. Hence, in an attempt to determine whether or not springbok use photoperiod to cue reproduction, I investigated the following: the duration of the reproductive period, the degree to which the onset of oestrus in females is synchronous and whether or not the onset of oestrus is spontaneous, irrespective of the time of year.

If springbok are not seasonal, the reproductive period should be relatively long or even unlimited, oestrus should not be synchronous and females should come into oestrus spontaneously. Alternatively, if springbok are seasonal breeders, the reproductive period should be limited (and inflexible), oestrus should be synchronous and the onset of oestrus may be either spontaneous or induced by conspecific males or females.

Methods

The onset of ovulatory activity at puberty can be detected by determining when progesterone secretion increases because this occurs with the onset of luteal activity after ovulation. Sex hormones can be measured in a number of bodily secretions/excretions, including blood

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(Hess *et al.* 1983), urine (Brand 1981), faeces (Bamberg *et al.* 1984) and saliva (Premawardhana *et al.* 1997). Due to the practical difficulties inherent in collecting excretory material frequently from a number of animals in a confined space, blood collection was the method of choice in this study.

Once cyclic activity has commenced, progesterone levels are high during the luteal phase and low during the follicular phase (Figure 12). If cyclic activity continues regularly and spontaneously, a regular pattern of progesterone secretion is produced. To determine the length of the luteal and follicular phase of the cycle, the interval between blood samples should be equal to or greater than the duration of the shorter of the two phases, this normally being the follicular phase. Liversidge & De Jaager (1984) reported that the average oestrous cycle length of a single springbok ewe was 16 days (range: 14 – 17). Thus, assuming that the follicular phase is about 20 % of the entire cycle length (Perry 1970), blood samples taken at a frequency of every 3 to 4 days should ensure that the nadir in progesterone secretion which occurs in the follicular phase is detectable against the otherwise higher levels of progesterone that characterise the luteal phase. In calculating the cycle length and the concentration of progesterone during a cycle, only data that unambiguously represented an oestrous cycle were used.

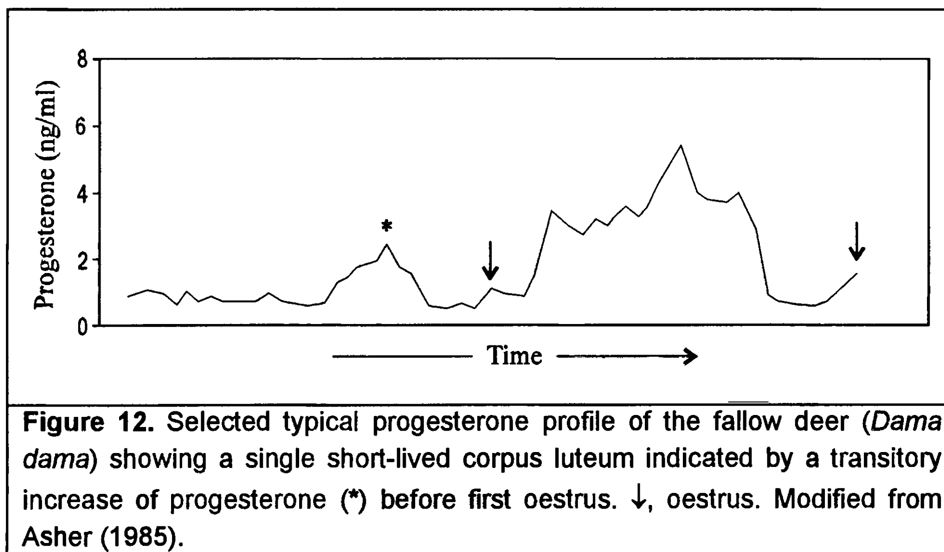

Figure 12. Selected typical progesterone profile of the fallow deer (*Dama dama*) showing a single short-lived corpus luteum indicated by a transitory increase of progesterone (*) before first oestrus. ↓, oestrus. Modified from Asher (1985).

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Sunderland *et al.* (1995) defined the onset of puberty in lambs as "the date of the first progesterone value above 0.5 ng/ml in a series of three or more samples above 0.5 ng/ml that included one above 1 ng/ml". However, the use of a threshold value to define the onset of puberty was inappropriate for this study because the luteal phase levels of progesterone are not known for springbok. For example, the threshold method could not distinguish between transitory increases in progesterone secretion and genuine oestrous cycles (see Figure 12, p. 42). Thus, the definition of the onset of cyclic activity used in this study is the date after which three progesterone values are greater than twice the mean of all preceding values. The duration of reproductive activity was defined as the number of days since the initiation of the first cycle to the termination of the last cycle, the last cycle being that cycle after which the next 10 values are indistinguishable from the mean of the values preceding cyclic activity.

To determine the onset of oestrus and the length of the reproductive period, a captive herd of springbok ewes (n = 9) was maintained and blood samples were collected twice weekly and assayed for progesterone.

Progesterone assay

Progesterone was extracted from plasma samples prior to radioimmunoassay. The extracted samples were assayed using standard radioimmunoassay techniques. The mean non-specific and total binding of 12 progesterone assays was 5.4 ± 0.3 and 30.7 ± 2.3 %, respectively. The mean intra- and inter-assay CV was 15.6 and 23.5 %, respectively. The average sensitivity was 32.4 ± 10.4 pg/ml. Further details—including the assay validation—are presented in Chapter 2.

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Results

The onset of oestrus in captivity

The progesterone profile of each animal is presented in Figure 13. Oestrus occurred spontaneously in all ewes. Seven of the nine animals commenced cyclic activity between 28 May and 30 July. Although the group as a whole was not synchronous, three ewes commenced cyclic activity between 11 and 25 June, two ewes commenced cyclic activity on 28 May and another two on 26 and 30 of July. 7 of 9 animals (78 %) commenced cyclic activity within 3 months of each other. The mean cycle length was 16.6 ± 0.6 days. The mean length of the luteal and follicular phases was 13.1 ± 0.3 and 3.5 ± 0.4 days, respectively. The mean concentration of progesterone was 3.86 ± 0.54 ng/ml in the luteal phase and 1.07 ± 0.36 ng/ml in the follicular phase. Although the ratio of luteal phase progesterone concentration to follicular phase concentration varied widely between animals, the concentration during the luteal phase was, on average, 3.6 fold higher than during the follicular phase (range: 1.9 – 11.9 times).

Influence of body mass on puberty

The weight of each animal over the course of the study is presented in Figure 14. The commencement of cyclic activity was not related to body weight in any obvious way: those animals that commenced cyclic activity almost synchronously in June ranged in mass from 18 to 21.5 kg, and the range in weight at which puberty was attained was 17 – 22.5 kg. Although the animals seemed to commence cyclic activity when their weight reached an asymptote (Figure

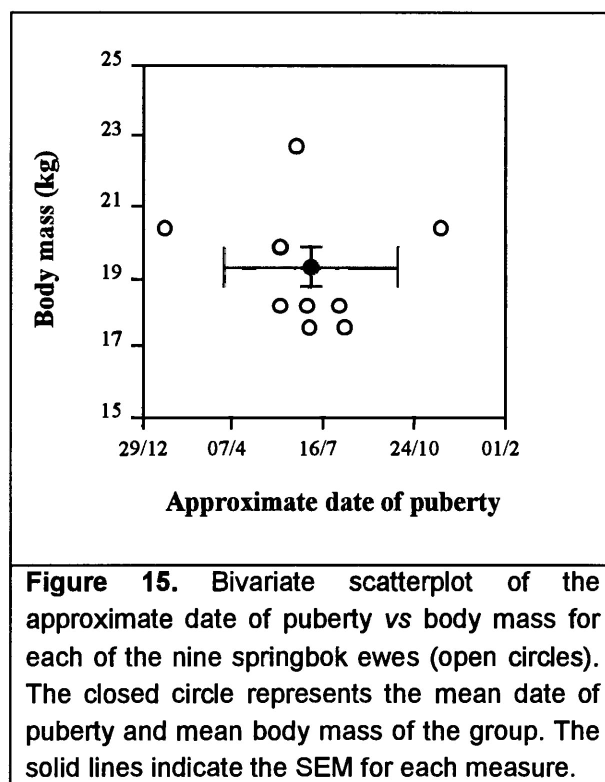

Figure 15. Bivariate scatterplot of the approximate date of puberty *vs* body mass for each of the nine springbok ewes (open circles). The closed circle represents the mean date of puberty and mean body mass of the group. The solid lines indicate the SEM for each measure.

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14), there was no relationship between the date on which puberty occurred and the weight of the animals (Figure 15). However, if there is a critical weight threshold beyond which animals enter puberty, this is probably greater than 18 kg, as only one animal reached puberty at a weight lower than this.

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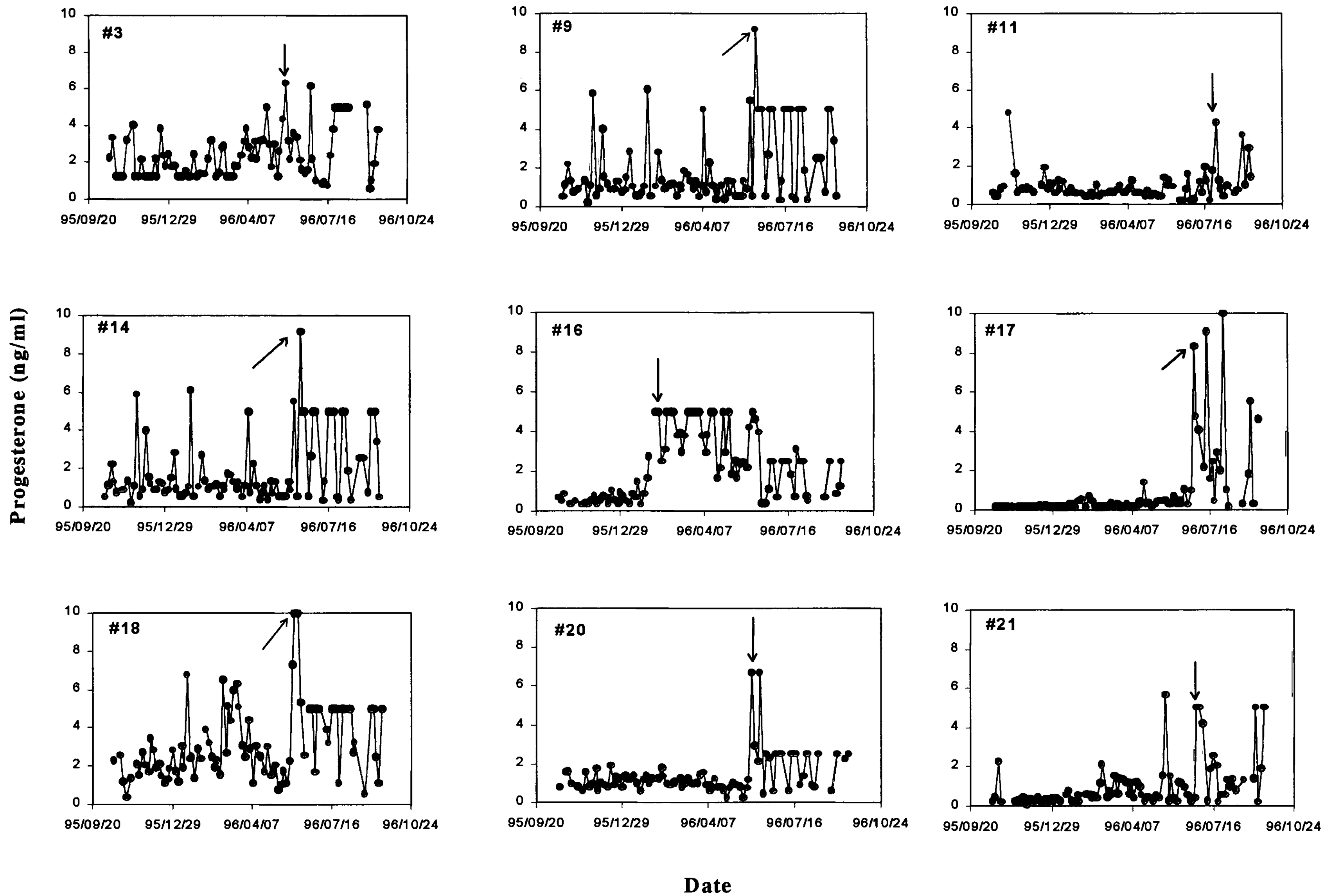

Figure 13. Progesterone profiles of the nine springbok ewes over the course of the study. Arrows indicate the onset of cyclic activity.

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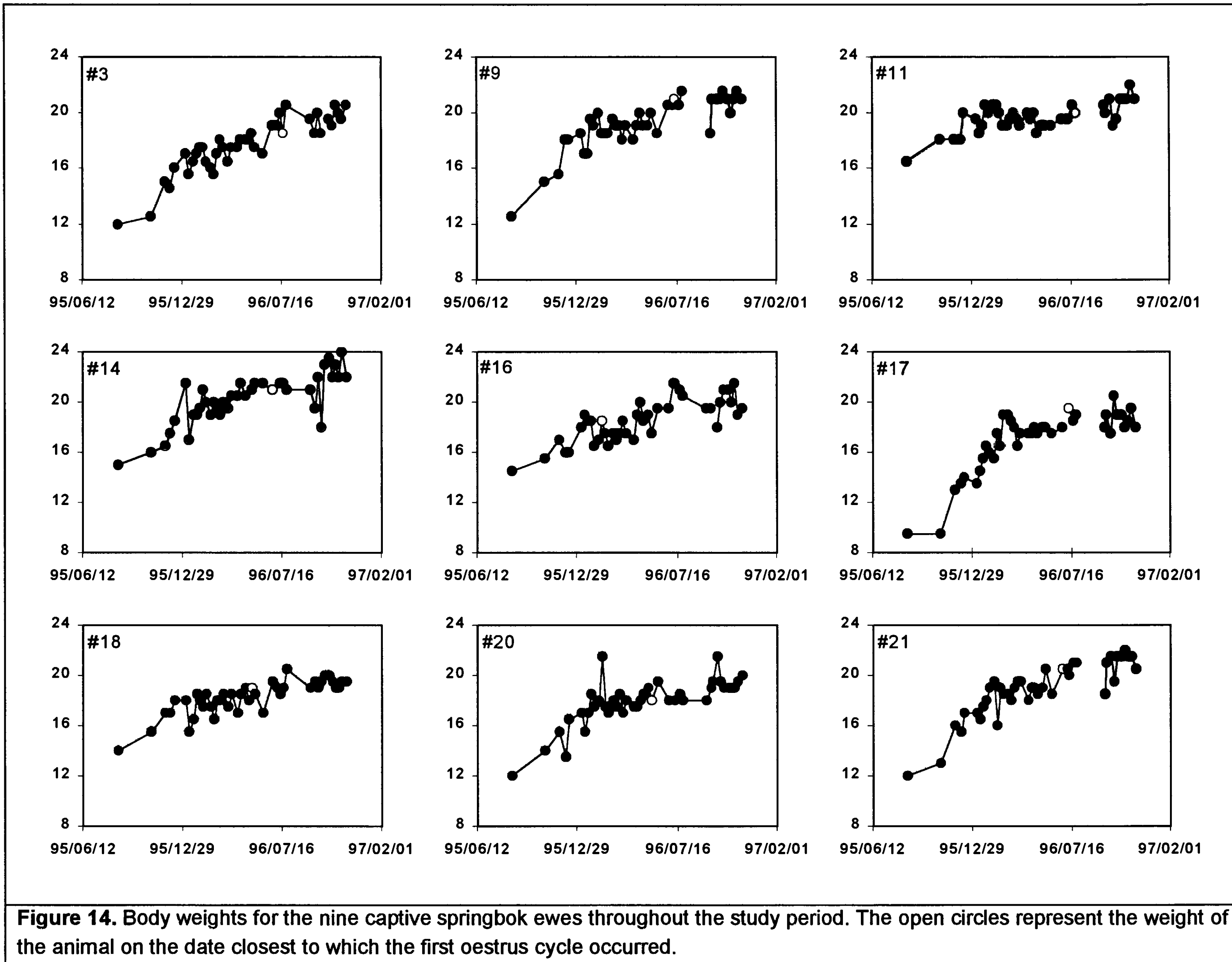

Figure 14. Body weights for the nine captive springbok ewes throughout the study period. The open circles represent the weight of the animal on the date closest to which the first oestrus cycle occurred.

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Discussion

The springbok observed in this study did not come into oestrus synchronously and lighter (younger) animals did not delay reproduction until the next season. Furthermore, the reproductive period was not short. Although it was not possible to test inter-annual variation in the length of the reproductive period in this study, sampling has continued and should provide insight into the degree to which the length of the reproductive period is flexible. Nevertheless, these observations do not support the hypothesis that springbok are seasonal. Thus, in captivity, in the absence of predation pressure and with constant nutrition and favourable environmental conditions, springbok show little sign of restricted or seasonal reproduction. However, while the results do not support seasonal breeding, they are not completely consistent with aseasonality either. For instance, the facts that some animals attained puberty at almost the same time and 78 % of the group attained puberty within 3 months of each other, suggest that springbok are restricted breeders to some degree. However, this suggestion should be tempered by the possible confounding effect of body weight on puberty. For instance, the majority of the study animals reached puberty at a similar body weight. While there was no significant effect of body weight on the date of puberty, there does seem to be a critical mass threshold beneath which cycling does not commence. Thus, the similarity in the dates at which puberty was reached may be largely due to the effects of body weight rather than a seasonal response.

Only when the physical (i.e. nutritional) needs of the animal have been satisfied is it in a position to respond differentially to environmental cues. For example, puberty in normally developing sheep is thought to be controlled by photoperiod rather than growth (Foster *et al.* 1988). However, some slow growing lambs may not attain puberty in the first breeding season when photoperiod normally stimulates reproduction; such animals do not attain puberty until the next breeding season, by which time they are sufficiently large. Indeed, delayed puberty from malnutrition is common in animals living in the wild (Bronson 1989). Conversely, some rapidly growing lambs do not attain puberty at the normal size when day length is increasing (I'Anson *et al.* 1991). This suggests that the interaction of internal and external cues that determine the onset of puberty is complex.

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Thus, in conclusion, it is suggested that springbok do not employ environmental variables such as photoperiod as cues to time the onset of reproductive activity. However, the existence of photoperiodic adaptations cannot be discounted. For instance, a number of putatively non-photoperiodic mammals have been found to be latently photoperiodic (Goldman & Nelson 1993). One reason for a lack of reproductive seasonality might be the absence of the physiological mechanism that is used to translate photoperiodic information into a neurochemical signal. This forms the subject of Chapter 5.

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Chapter 5

Is the pineal-melatonin system intact in springbok?

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Introduction

The results presented in Chapter 3 and 4 suggest that springbok are not strictly photoperiodic, which is the case in most species that do not reproduce seasonally (Goldman & Nelson 1993). The question now arises, why are springbok in particular, and aseasonal species in general, not strictly photoperiodic?

There are two hypotheses that arise from the consideration of the apparent lack of photoperiodicity in aseasonal animals. First, aseasonal animals might lack a functional pineal-melatonin system, which would be manifested by the absence of a normal rhythm of melatonin secretion. However, while this hypothesis is plausible, it is unlikely to be true as melatonin rhythms have been reported in every mammal examined to date (reviewed by Arendt 1995). Another problem with this hypothesis is that it fails to account for the possible absence of a melatonin signal. It is possible that a melatonin signal might be absent because there is no selective pressure for its maintenance. Alternatively, the organism may never have had a melatonin rhythm to begin with and thus may never have been in a position to evolve photoperiodicity.

An alternative hypothesis is that aseasonal animals do possess a functional pineal-melatonin system but fail to respond to its signal because this would be maladaptive. This hypothesis implies that aseasonality is not due to a lack of photoperiodic potential, but rather that there is an adaptive advantage to being non-photoperiodic.

The pineal-melatonin system of springbok was examined to ascertain whether this system exists and functions normally in an aseasonal ungulate.

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Methods

Animals

A herd of springbok (n = 9 females & 2 males) was maintained in captivity near Pretoria, South Africa (25°45'S 28°10'E). The experiment was performed in spring (11 – 14 October) under natural photoperiodic conditions. The animals were habituated to the handling routine for one month prior to the experiment. They were captured at various times (see Figure 16) of the day and night over a period of four days. The animals were restrained by hand and no sedatives were used (see Appendix 1, p. 84). On each occasion, 5 ml of blood was collected from the jugular vein by venepuncture. Blood was processed as described in Chapter 6 (p. 55). During the sampling procedure, care was taken not to expose the animals to any direct light. Accordingly, they were blindfolded and a weak (intensity *c* 15 lux) red-filtered lamp was used to facilitate blood sampling.

Melatonin assay

Melatonin was radioimmunoassayed in duplicate 100 μl aliquots of plasma using the method of Fraser *et al.* (1983) with an Ab first raised by Tillet *et al.* (1986). All samples were analysed in a single assay, for which the intra-assay CV for 2 plasma pools averaged 9.1 %. Sensitivity was 16.0 pg/ml.

Results

With the onset of darkness, the plasma concentration of melatonin increased significantly (ANOVA, $p < 0.01$, $n = 11$) from the average daytime value of 17.7 ± 3.9 pg/ml. Nocturnal concentrations averaged 115.0 ± 15.5 pg/ml. The concentration of melatonin varied significantly between animals (ANOVA, $p < 0.0001$, $n = 11$) with the widest range in values occurring at night (10.0 ± 1.9 – 348.0 ± 50.2 pg/ml).

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The profile of plasma melatonin concentration was bimodal with a pronounced nadir midway through the night (Figure 16). This nadir was not due to variation in the concentration of melatonin in individual animals; rather, each animal (except animal no. 3) showed a similar pattern.

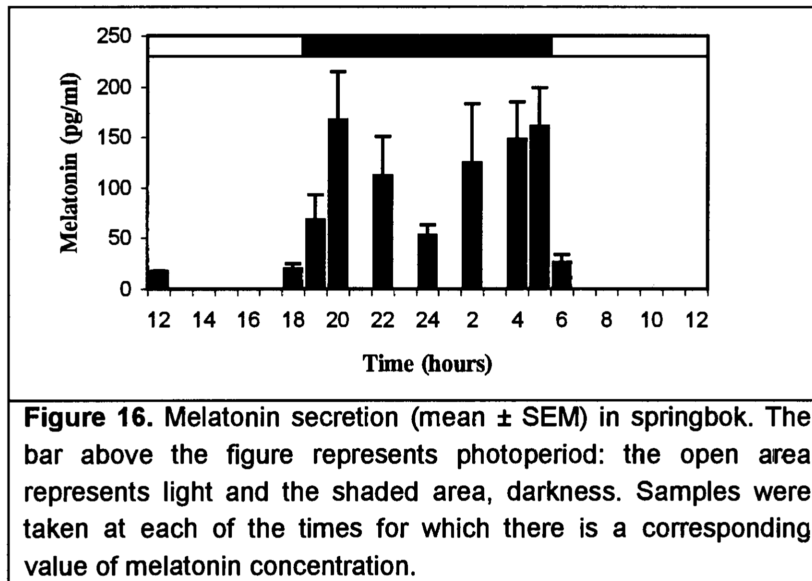

Figure 16. Melatonin secretion (mean ± SEM) in springbok. The bar above the figure represents photoperiod: the open area represents light and the shaded area, darkness. Samples were taken at each of the times for which there is a corresponding value of melatonin concentration.

Discussion

The mean night time levels of melatonin in springbok are comparable to those of other mammals. As in most mammals, there was great variation between individuals in the concentration of plasma melatonin. Since selection reduces variation (Price 1995), large variation in a trait is evidence of a lack of selective pressure acting upon it. It is thus tempting to infer that the variation in plasma melatonin of springbok is evidence of a lack of selective pressure on this trait, which in turn implies that the melatonin rhythm is a relatively unimportant parameter of fitness. However, in contrast to the variability in the concentration of plasma melatonin, the duration of melatonin secretion appears to be less variable. Thus, while the absolute concentration of melatonin may be a relatively unimportant fitness parameter, this may not be the case for the duration of melatonin secretion.

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A nadir in melatonin concentration at midnight was exhibited by all but one of the springbok. This might simply be an artefact of the sampling regime. For example, the natural moonlight on the night when the 24h00 sample was taken might have been brighter than on other occasions and depressed melatonin secretion at this time. Indeed, Arendt (1995) noted that bright moonlight depressed the concentration of melatonin in sheep. The profile in Figure 16 and that of sheep exposed to a pulse of light at night are strikingly similar. In the latter study, sheep that were exposed to 1 h of light in the middle of a short night showed short-day responses (Rauvault & Thimonier 1988). This suggests that the phase corresponding to the time when the light pulse occurred might be a sensitive phase in which the presence of melatonin can induce a short-day response (Malpaux *et al.* 1993). How this would function is not immediately apparent; however, the compression of each of the melatonin secretion phases (the pre- and post-midnight secretion phases) in long days could cause an overlap that results in a monophasic plasma melatonin profile without a decrease in melatonin concentration at midnight. Thus, the presence of a nocturnal nadir in short days, and its possible absence in long-days, could conceivably provide a clearly differential and interpretable melatonin signal.

The concentration of plasma melatonin in springbok is clearly affected by light. The pineal-melatonin system is thus present and apparently functional in springbok. However, springbok are not strictly seasonal breeders and cannot be said to be photoperiodic in their natural habitat (Chapter 3 and 4, p. 31 & 40). Thus, the hypothesis that springbok are aseasonal because they lack a functional pineal-melatonin system, is rejected. This suggests that springbok may not have evolved an ability to use photoperiodic cues even though they possess the physiological capacity for photoperiodism. This may be applicable to a broader range of taxa that share similar characteristics, i.e. an intact pineal system and aseasonal reproduction. For instance, some populations of white-footed mice (*Peromyscus leucopus*) and deermice (*P. maniculatus*) are non-photoperiodic, yet have the same melatonin rhythms as mice from photoperiodic populations (Lynch *et al.* 1982, Dark *et al.* 1983). While further research into related species should provide more insight into this phenomenon, the physiological basis for the lack of photoperiodic responsiveness is suggested to lie downstream from the pineal-melatonin system.

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Chapter 6

What changes occur in the secretion of LHRH and neurotransmitters during the transition from reproductive quiescence to activity?

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Introduction

In all mammals, the secretion of LHRH is essential for the sustained secretion of gonadotrophins (Knobil 1980, Valk *et al.* 1980). By extension, virtually all reproductive processes are ultimately dependent on the proper functioning of the neuronal systems regulating LHRH secretion. The biological necessity of LHRH secretion is easily demonstrated: if the actions of LHRH are blocked by immunoneutralisation or receptor antagonism (Ellis *et al.* 1983, Grady *et al.* 1985), the result is reduced gonadotrophin secretion, disrupted gonadal function and infertility.

The preceding chapters have demonstrated that the physiological system that underlies reproduction in seasonally breeding mammals is also present in springbok. Furthermore, the neuroendocrine axis comprising LHRH, gonadotrophins and ovarian hormones is the same in all ungulates. This suggests that the neural mechanisms that are responsible for activating the LHRH system with the transition from reproductive quiescence to oestrus, are likely to be similar. However, it is not possible to investigate the neuroendocrine system that underlies the initiation of reproductive behavior in springbok because the technical facilities are not available. Thus, the sheep is an excellent alternative model to springbok for investigating the transition from reproductive quiescence to oestrus as it is the Bovid about which the most is know regarding the neural and endocrine regulation of reproduction.

The effects of photoperiod on reproductive state are well established in sheep. A short-day photoperiod or short-day like melatonin treatment stimulates LH secretion in anoestrous ewes after a delay of 40 – 60 days (Bittman *et al.* 1985, Viguié *et al.* 1995*a*). However, the lag in the stimulation of LH is due to a delay in the stimulation of LHRH secretion and not LH secretion (Viguié *et al.* 1995*a*). Thus, melatonin acts by altering LHRH secretion rather than altering the pituitary responsiveness to LHRH. Thus, the endocrine changes associated with photoperiodically induced alterations of reproductive state are secondary to changes in hypothalamic function (Steger *et al.* 1982, Viguié *et al.* 1995*a*).

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While it is clear that melatonin is responsible for modulating the transitions in reproductive state via the modulation of LHRH secretion, no direct link has been established between melatonin and the LH-LHRH neurosecretory system. For instance, melatonin microimplants placed in the preoptic area, where the majority of the LHRH cell bodies are located, does not affect LH secretion (Malpaux *et al.* 1993*b*). Also, melatonin implanted near the pars tuberalis, which has the highest density of melatonin binding sites (Morgan *et al.* 1989, Bittman & Weaver 1990), fails to produce short-day effects (Malpaux *et al.* 1994). Thus, a system of interneurones is suspected to bridge the gap between the sites of melatonin action and LHRH neurones. Neurotransmitters such as excitatory amino acids and catecholamines have all been implicated in the melatonin-mediated control of LHRH secretion. Thus, these substances are good candidates for forming part of a sequence of neurotransmission events that indirectly link melatonin to the modulation of LHRH secretion.

Inhibitory neurotransmitters are responsible for the decreased secretion of LH and LHRH observed in the anoestrous season (Meyer & Goodman 1985, Goodman 1989). The reestablishment of LH secretion in the breeding season is associated with decreased dopaminergic activity (Viguié *et al.* 1993) and excitatory amino acid secretion has also been implicated (Lincoln & Wu 1991). 5-HT is generally acknowledged to have an inhibitory action on LH secretion in sheep (Domanski *et al.* 1975), although it may be stimulatory or permissive in other species (e.g. rats, Frankfurt 1981) or have no discernable role at all (e.g. Syrian hamsters, Steger *et al.* 1984).

Previous studies have established that changes in neurotransmission do occur with the switch from anoestrus to oestrus. The aim of this study was to elucidate the changes in the secretion of neurotransmitters relative to LHRH secretion in ovariectomised, oestradiol-treated ewes to determine whether there are changes in neurotransmitters that can be linked to the photoperiodically induced stimulation of LHRH secretion. Furthermore, the form that the increase in LHRH secretion takes was assessed to determine whether the increase in LHRH secretion is gradual or discrete.

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Materials & Methods

Animals

Experiments were conducted on sexually mature Ile-de-France ewes (*n* = 20) at Nouzilly, France. All ewes were housed in rooms with free access to water and were fed daily with hay, straw and corn. During the experiment, the ewes were able to move forwards and backwards but were unable to turn around. They were always in contact with other animals. All the ewes (*n* = 20) were ovariectomised at least one week prior to experimentation. Each animal received a subcutaneous oestradiol implant at the time of ovariectomy. All of the animals were surgically cannulated (see below) and held in long days (16L:8D) until Day 0 of the experimental protocol.

Ovariectomy and oestradiol implantation

Anaesthesia was induced with pentothal (10mg/kg body weight) and maintained with halothane (5% in oxygen, 700 ml/min). A bilateral ovariectomy was performed via mid-ventral laparotomy. While the ovariectomy was being performed, a homemade implant, made from a Silastic tube filled with about 20 mm of crystalline oestradiol-17ß (Karsch *et al.* 1973), was implanted subcutaneously. The concentration of oestradiol released from the implants was calculated to simulate luteal phase levels of oestrogen secretion (3 – 4 pg/ml, Karsch *et al.* 1987). The implants were pre-incubated overnight in water (25°C) to minimise transient post-implantation steroid release.

CSF collection and processing

To collect CSF, the animal was restrained manually while the cap of the Teflon housing apparatus was opened. The inside of the housing was sterilized with alcohol and the cannula stopper removed. This stopper was replaced by a stopper of the same dimensions through which a sterile silastic catheter (Giotrol, Paris, France) had been passed (outside ∅ = 0.7 mm; inside ∅ = 0.3 mm). The catheter was fixed to the stopper in a position so that the tip

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projected 3 mm beyond the end of the guide cannula, into the third ventricle. Approximately 150 mm of catheter was left attached to the stopper on the outside. If CSF did not flow from the catheter of its own accord, a 1 ml syringe fitted with a sterile 26 gauge needle was attached to the external end of the catheter. CSF was then slowly aspirated from the catheter. When CSF was observed to flow into the syringe, the needle was detached from the catheter and CSF was allowed to flow of its own accord by gravity.

If CSF did not flow of its own accord, the stoppered and attached catheter were removed from the cannula and a sterile stylet was inserted into the cannula in an attempt to clear it of any obstruction (e.g. accumulation of glial cells). The stylet was constructed to fit snugly into the cannula and project approximately 1 mm beyond the cannula tip into the third ventricle. The stylet was removed and the catheter reinserted. In the event of CSF still not flowing, the catheter was slowly moved toward the outside by retracting the stopper. If CSF began to flow after retracting the catheter, a new catheter was prepared under sterile conditions that was 1 mm shorter than the other catheter. This procedure was repeated until a catheter of suitable length was obtained. If CSF still did not flow after using the stylet and maneuvering the catheter within the cannula, the animal was excluded from the experiment.

After collection of CSF was complete, the flow of CSF from the catheter was stopped by inserting a tight-fitting steel plug, constructed from 26 gauge steel wire, into the end of the catheter under sterile conditions. After this, the catheter was placed inside the Teflon housing and the housing cap was replaced. The catheter could be left in place without any apparent ill effects on the animal. Only occasionally were flow problems encountered (probably due to accumulation of glial cells within the cannula/catheter), although the presence or absence of a catheter seemed to have little effect on whether or not this occurred.

For the intensive sampling occasions (Day – 4 to – 1 and Day 74 and 75), an integrated CSF sample was collected by attaching the free end of the catheter to approximately 2.5 m of polyethylene tubing of the same diameter (Anachem, Luton, UK). The peristaltic tubing was threaded through a rate-adjustable peristaltic pump (Minipuls 2, Gilson, Villiers-le-bel, France) and CSF fractions were extracted for the desired period. All connections between the tubing

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were made using 26 gauge needles (Microlance, Becton-Dickinson, Dublin, Ireland) which had been blunted at both ends. For each animal, the time taken for the extracted CSF to reach the fraction tubes and the volume of CSF collected per unit time was recorded. This provided an estimate of the true time that the sample was extracted from the ventricle, the actual concentration of LHRH in the CSF and the collection rate.

For determination of LHRH in the intensive collections, CSF was collected directly into methanol (2 ml). After the CSF had been collected, it was vortexed and centrifuged at 4°C. The supernatant was then decanted into clean glass tubes and subsequently evaporated at room temperature while being centrifuged in an evacuated chamber (Speedvac, Savant, Faringdale, USA). The dried tubes, which contained the LHRH-bearing residue, were kept frozen at – 20°C until the LHRH radioimmunoassay.

For determination of neurotransmitters collected during the intensive sampling occasions, CSF was collected into Dounce buffer (50 μl). These samples were then split into two 300 μl samples and kept frozen at – 80°C until the concentrations of the neurotransmitters were assessed using high pressure liquid chromatography (HPLC).

The bi-weekly CSF samples were handled as follows. Approximately 600 μl of CSF was collected from the distal end of the catheter on each sampling occasion. 500 μl of this CSF was then pipetted into 2 ml of methanol and the remaining 100 μl into a tube containing 50 μl Dounce buffer. The samples were then treated as above.

Blood collection and processing

Instantaneous blood samples were collected through an indwelling jugular catheter. The blood and CSF samples were collected simultaneously by taking the blood sample immediately at the end of each CSF sampling period. Blood was centrifuged (4°C), plasma harvested and stored at – 20°C until the radioimmunoassay for LH was carried out.

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Radioimmunoassays

LH was assayed in duplicate 100 μl aliquots of plasma using the radioimmunassay method of Pelletier *et al.* (1968), modified by Montgomery *et al.* (1985). All samples were analysed in a single assay, for which the intra-assay CV for 5 plasma pools averaged 6.5 %. Sensitivity was 0.13 ng/ml of 1051-CY-LH.

LHRH concentration was determined after extraction of CSF in 2 ml methanol. Recovery of LHRH after extraction was > 80 %. LHRH was assayed in single aliquots corresponding to 200 μl of plasma using the method of Caraty & Locatelli (1988). The intra-assay CV for 2 plasma pools was 13.8 %, and the interassay CV was 10.6 %. Sensitivity was 0.63 pg/ml.

HPLC (Pump Spectra Physics SP8810, autosampler CMA 2000) and electrochemical detection (Waters model M460) were used to quantify Asp, Glu, GABA, DOPAC, 5-HT and 5-hydroxyindoleacetic acid (5-HIAA). The mobile phase consisted of 8.5 % (mass/volume) KH_2PO_4 and 5 % (volume/volume) MeOH in distilled water adjusted to pH 4.2. The flow rate was 1.2 ml/min. The limits of detection for each sample were defined as the quantity giving a peak with an amplitude equal to twice the amplitude of the baseline noise (Gallegos-Sanchez *et al.* 1996).

Data analysis

For each animal, the neuroendocrine axis was considered to have been activated at the time that the first of at least three bi-weekly sample values was greater than 1 ng/ml (Viguié *et al.* 1995*a*, *b*).

Experimental design

The experimental design is illustrated in Figure 17. Experiment I was designed to examine the effects of long-term CSF-sampling on the neuroendocrine axis. To this end, the concentration

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of LH, LHRH and selected neurotransmitters (see p. 61) was compared between ewes that had CSF sampled bi-weekly for 11 weeks and ewes that were not sampled.

The concentration of LH, LHRH and selected neurotransmitters was first determined in ewes that had low levels of LH. Samples were collected over four days (from Day −4 to Day −1). On Days −4 and −2, CSF and blood was sampled from 10 animals for six hours. CSF was withdrawn from the third ventricle via the catheterised guide cannula, using a peristaltic pump, at a rate of 30 μl/min. This allowed an integrated sample of about 0.3 ml to be collected every 10 min. The CSF was collected directly into 2 ml of methanol. Blood (5 ml) samples were collected every 10 min and held on ice until centrifugation. The CSF collected on these days

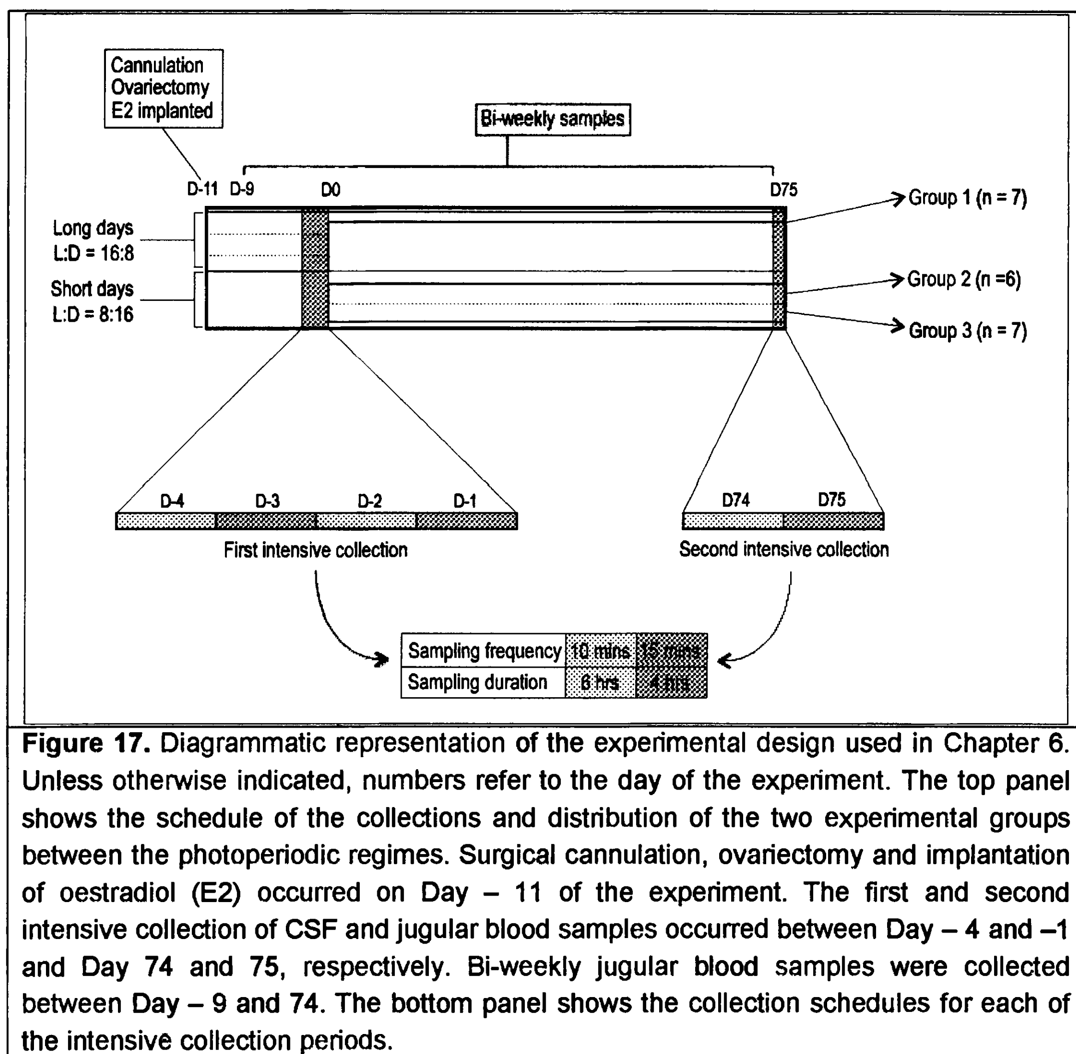

Figure 17. Diagrammatic representation of the experimental design used in Chapter 6. Unless otherwise indicated, numbers refer to the day of the experiment. The top panel shows the schedule of the collections and distribution of the two experimental groups between the photoperiodic regimes. Surgical cannulation, ovariectomy and implantation of oestradiol (E2) occurred on Day – 11 of the experiment. The first and second intensive collection of CSF and jugular blood samples occurred between Day – 4 and –1 and Day 74 and 75, respectively. Bi-weekly jugular blood samples were collected between Day – 9 and 74. The bottom panel shows the collection schedules for each of the intensive collection periods.

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was later assayed for LHRH. On Days –3 and –1, CSF and blood was collected from the same ewes for four hours. CSF and blood samples were collected every 15 min. The concentration of the neurotransmitters was determined in the CSF collected on these days.

After the completion of this sampling session, the ewes were divided into three groups on what was designated as Day 0 of the experiment. Group 1 (n = 7) was maintained in long days (16L:8D) and groups 2 (n = 6) and 3 (n = 7) were transferred to a short-day photoperiod (8L:16D); group 1 thus acted as a control for the effects of photoperiod. Groups 1 and 2 were both subjected to bi-weekly CSF and blood collection until they showed increased LH secretion (about 11 weeks). This was confirmed by a radioimmunoassay of LH. CSF was not sampled from animals in group 3, thereby providing a control for the effects of CSF withdrawal. The collection procedure used on Days – 4 to – 1 was then repeated on Days 74 and 75 on ewes in which LH levels were high. Data from animals in group 2 represented CSF-sampled animals while group 3 acted as a control for the effects of CSF-withdrawal.

Experiment II was designed to examine changes in the secretion of LH, LHRH and neurotransmitters and to determine what form the increase in LH and LHRH secretion takes after photostimulation of the neuroendocrine axis. Data from experiment I was used. The data for animals in groups 2 and 3 were combined to represent a photostimulated group while group 1 acted as a photo-inhibited control.

Results

Recovery & Survival

All animals (n = 20) recovered from the surgical procedure. After the conclusion of the experiment (Day 75), 16 of the 20 animals were still alive. CSF collection was still possible in 10 (63 %) of these animals.

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Part I: Assessment of CSF sampling method

Effect(s) of long-term CSF withdrawal

LH and LHRH

On Days – 4 to -1, the CSF-sampled and unsampled groups were very similar with respect to LH and LHRH secretion; On Days 74 and 75, the two groups still had similar LH concentrations (Figure 18). Both groups had elevated levels of LH (Figure 18). The mean concentration of LHRH as elevated in the unsampled animals but lower in the CSF-sampled animals (Figure 19). A statistical analysis was not possible due to the small sample sizes.

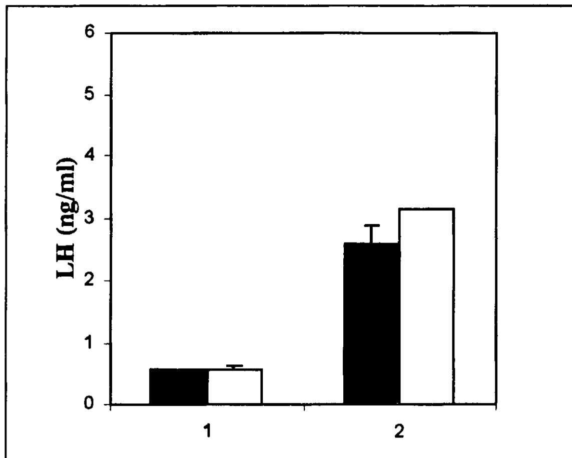

Figure 18. LH secretion in CSF-sampled (solid bars) and unsampled (open bars) ewes. 1, low pulsatile situation. 2, high pulsatile situation. All values expressed as mean ± SEM.

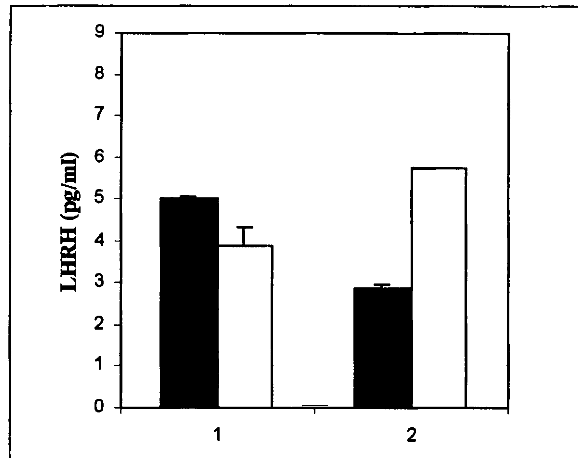

Figure 19. LHRH secretion in CSF-sampled (solid bars) and unsampled (open bars) ewes. 1, low pulsatile situation. 2, high pulsatile situation. All values expressed as mean ± SEM.

Neurotransmitters

The results of the determinations of neurotransmitter concentrations are presented in Figure 20. Although the CSF-sampled and unsampled groups could not be compared statistically due to small sample sizes, the difference in the concentration of Asp, Glu and GABA between the two groups on Days – 4 to – 1 was 70, 11 and 68 %, respectively. On Days 74 and 75, the

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difference was 47, 26 and 3 %, respectively. Thus, the difference in Asp concentration between the groups decreased by 33 % between the two sampling sessions, while Glu increased by 143 % and GABA decreased by 96 %.

In the CSF-sampled group, Asp secretion decreased from 44.9 ± 9.3 (n = 3) to 29.4 ± 4.9 (n = 3) ng/ml (36 %), Glu decreased from 1015.6 ± 126.0 (n = 4) to 693.3 ± 91.2 (n = 3) ng/ml (32 %) and GABA decreased from 852.13 ± 37.09 (n = 3) to 540.1 ± 83.5 (n = 3) ng/ml (37 %). In the unsampled group, Asp secretion decreased from 76.5 to 15.5 ng/ml (394 %), Glu decreased from 1123.5 to 515.0 ng/ml (118 %) and GABA decreased from 1433.3 to 523.3 ng/ml (174 %). Thus, both groups showed decreased secretion of each of the neurotransmitters examined. These degrees of difference with respect to the changes in secretion could not be tested for statistical significance; however, the decreased secretion observed in the unsampled animals is greater (range = 118 – 394 %) than the same parameters in the CSF-sampled group (range = 32 – 37 %).

The difference in the concentration of DOPAC, 5-HIAA and 5-HT between the two groups on Days – 4 to – 1 is not known because the data for animals in the unsampled group was lost. Similarly, the difference in the concentration of these molecules before and after CSF-sampling within the unsampled group cannot be calculated. However, the difference in the concentration of DOPAC, 5-HIAA and 5-HT between the CSF-sampled and unsampled animals on Days 74 and 75 was 10, 19 and 10 %, respectively. In the CSF-sampled group, DOPAC secretion decreased from 0.9 ± 0.1 (n = 4) to 0.6 ± 0.0 (n = 3) ng/ml (31 %). 5-HIAA secretion increased from 4.5 ± 0.4 (n = 4) to 18.7 ± 0.7 (n = 3) ng/ml (318 %) and 5-HT increased from 4.6 ± 0.2 (n = 4) to 16.1 ± 1.3 (n = 3) ng/ml (253 %).

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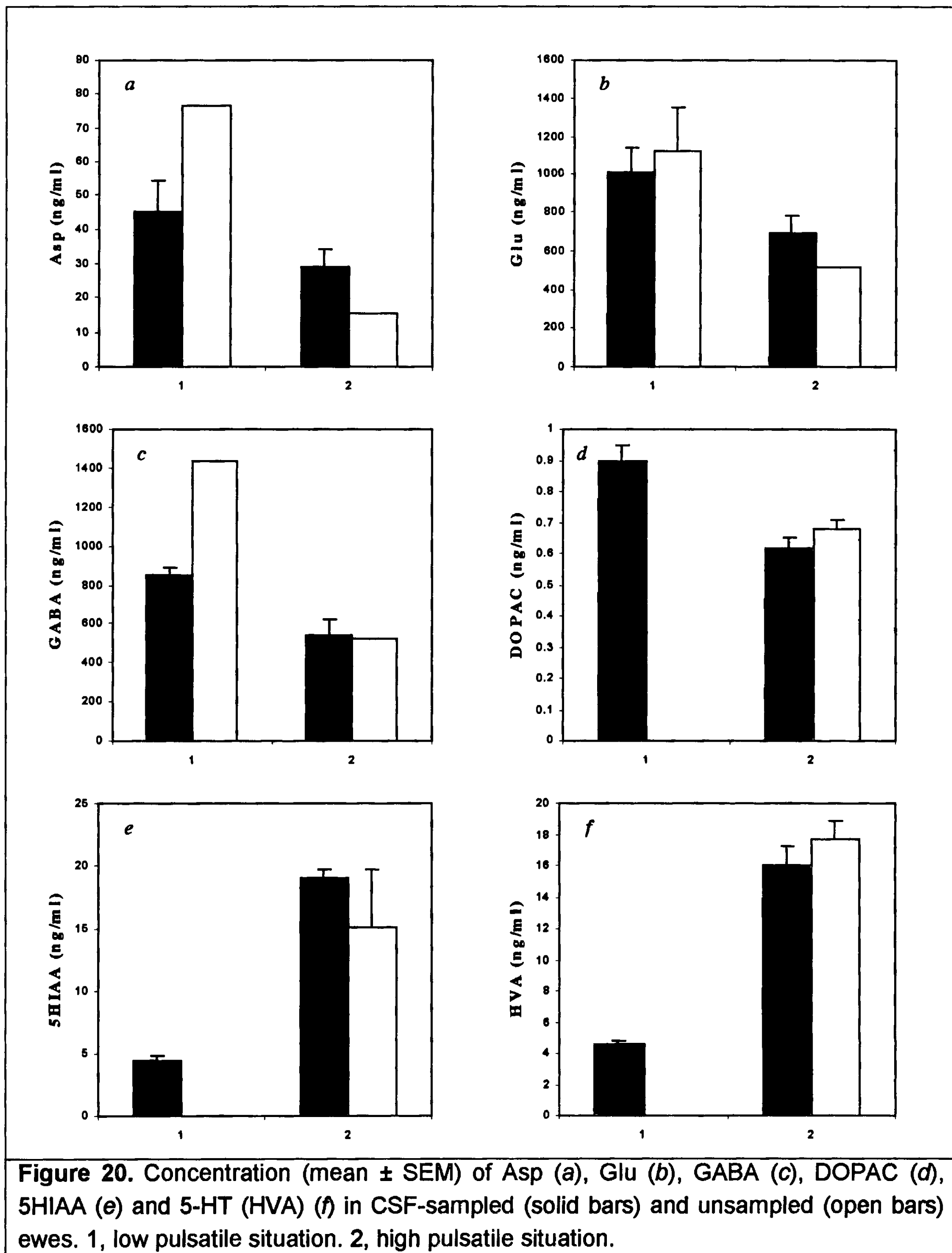

Figure 20. Concentration (mean ± SEM) of Asp (*a*), Glu (*b*), GABA (*c*), DOPAC (*d*), 5HIAA (*e*) and 5-HT (HVA) (*f*) in CSF-sampled (solid bars) and unsampled (open bars) ewes. 1, low pulsatile situation. 2, high pulsatile situation.

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Part II: The effect of photoperiod on the secretion of LH, LHRH & neurotransmitters

Detection of the change in LH & LHRH secretion

LH

The total concentration of LH secreted by ewes in the photostimulated group increased significantly from 0.6 ± 0.0 ng/ml (n = 9) on Days – 4 to –1 to 2.7 ± 0.5 ng/ml (n = 5) on Days 74 and 75 (Student's t-test, $p < 0.05$, Figure 21). The total concentration of LH secreted by the photo-inhibited ewes increased significantly from 0.5 ± 0.0 ng/ml (n = 7) on Days – 4 to -1, to 1.6 ± 0.4 ng/ml (n = 4) on Days 74 and 75 (Student's t-test, $p < 0.05$, Figure 21). The total concentration of LH was similar in both groups on Days – 4 to -1. However, LH levels seemed higher after photostimulation in the photo-stimulated ewes when compared to the photo-inhibited ewes, although a statistical test was not possible.

LHRH

No significant differences were detected in LHRH secretion after photostimulation (Student's t-test, $p > 0.05$ for all). The total concentration of LHRH secreted by the photostimulated ewes was 4.7 ± 0.3 pg/ml (n = 7) on Days – 4 to –1 and 3.8 ± 1.0 pg/ml (n = 3) on Days 74 and 75 (Figure 22). The small number of photo-inhibited ewes that provided data for the second sampling session (n = 2) prevents any statistical analysis of the data. Nevertheless, LHRH secretion was similar throughout the study. Specifically, the total concentration of LHRH secreted by the photo-inhibited ewes was 6.1 ± 1.5 pg/ml (n = 5) on Days – 4 to –1 and 8.4 ± 1.5 pg/ml (n = 2) on Days 74 and 75 (Mann-Whitney U-test, $p > 0.05$, Figure 22). Finally, at no stage of the experiment did the photostimulated and photo-inhibited groups differ significantly (Student's t-test, $p > 0.05$ for all) with respect to the total concentration of LHRH secreted (Figure 22).

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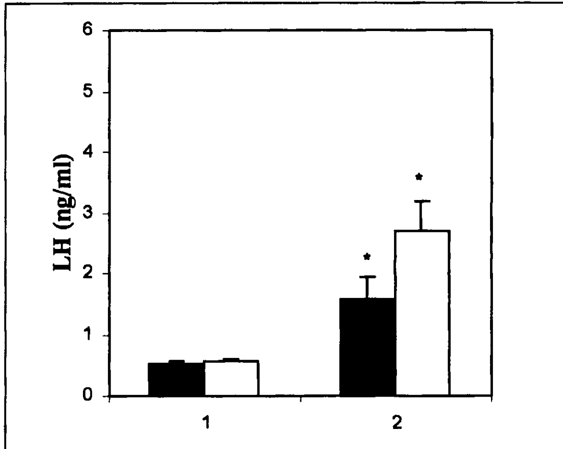

Figure 21. LH secretion in photo-inhibited (solid bars) and photostimulated (open bars) ewes. 1, low pulsatile situation. 2, high pulsatile situation. (*), significance within a group. All values expressed as mean ± SEM.

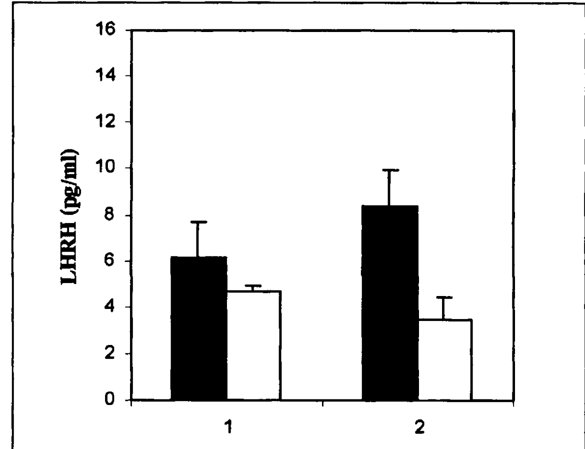

Figure 22. LHRH secretion in photo-inhibited (solid bars) and photostimulated (open bars) ewes. 1, low pulsatile situation. 2, high pulsatile situation. All values expressed as mean ± SEM.

Changes in neurotransmitter secretion

The results of the determinations of neurotransmitter concentrations are presented in Figure 23. The difference in the concentration of Asp, Glu and GABA between the photostimulated and photo-inhibited ewes on Days – 4 to – 1 was 36, 52 and 231 %, respectively. On Days 74 and 75, the difference was 189, 11 and 107 %, respectively. Thus, the difference in Asp concentration between the groups increased five fold between the two sampling sessions, while Glu decreased five fold. The difference in GABA secretion decreased two fold.

In the photostimulated group, Asp secretion decreased from 52.8 to 29.9 ng/ml (104 %), Glu decreased from 1051.6 to 648.8 ng/ml (62 %) and GABA decreased from 997.4 to 535.9 ng/ml (86 %). The change in Asp concentration could not be tested for significance, although the concentration was only half as great after photostimulation. The changes in Glu and GABA were also relatively great. In the photo-inhibited group, Asp secretion increased slightly from 72.1 to 74.9 ng/ml (1 %), Glu decreased from 1599.5 to 717.0 ng/ml (123 %) and GABA decreased from 3296.0 to 1104.7 ng/ml (198 %).

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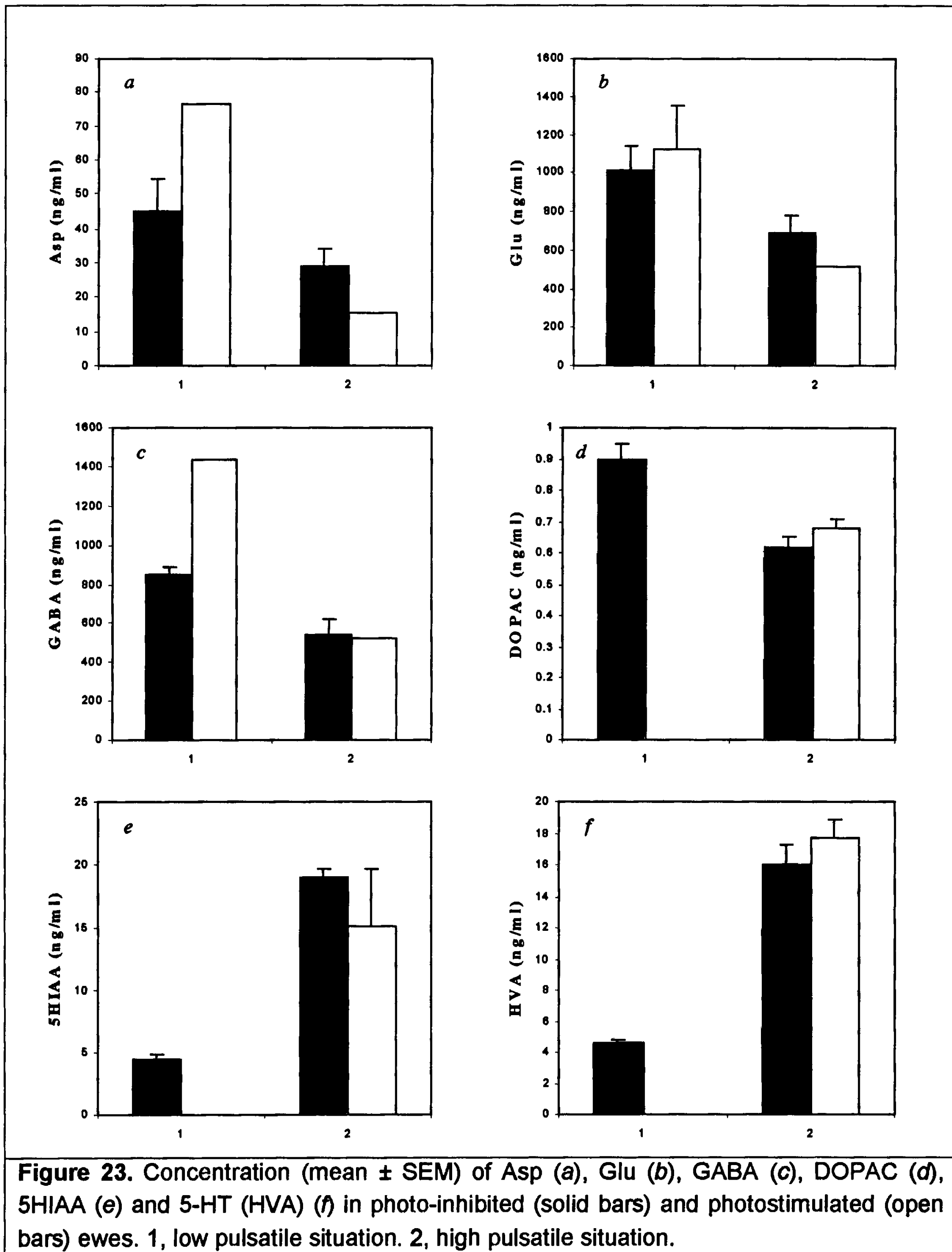

Figure 23. Concentration (mean ± SEM) of Asp (*a*), Glu (*b*), GABA (*c*), DOPAC (*d*), 5HIAA (*e*) and 5-HT (HVA) (*f*) in photo-inhibited (solid bars) and photostimulated (open bars) ewes. 1, low pulsatile situation. 2, high pulsatile situation.

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The difference in the concentration of DOPAC, 5-HIAA and 5-HT between the photostimulated and photo-inhibited ewes on Days – 4 to –1 was 7, 24 and 27 %, respectively. On Days – 4 to -1, the difference was 23.4, 16.8 and 16.9 %, respectively. Thus, the difference in DOPAC concentration between the groups increased 3 fold between the two sampling sessions, the difference in 5-HIAA concentration decreased slightly (by 8 %), and the difference in 5-HT concentration decreased by 10 %.

In the photostimulated group, DOPAC secretion decreased from 0.9 to 0.6 ng/ml (41 %), 5-HIAA increased from 4.5 to 17.2 ng/ml (286 %) and 5-HT increased from 4.6 to 16.7 ng/ml (267 %). The change in 5-HIAA could not be tested for significance, although it was also substantial. In the photo-inhibited group, DOPAC secretion decreased slightly from 0.8 to 0.8 ng/ml (6 %), 5-HIAA increased from 3.4 to 20.1 ng/ml (496 %) and 5-HT increased from 5.8 to 19.5 ng/ml (237 %).

Form of the change in LH and LHRH secretion

LH secretion in the photostimulated group clearly increased over time, in contrast to the consistently low levels of LH in the photo-inhibited group (Figure 24). The earliest increase in LH secretion occurred on Day 4 (ewe #5) and the latest on Day 62 (ewe #8) (Figure 26). Mean LH secretion in the photostimulated ewes exceeded 1 ng/ml by day 25 (1.1 ± 0.1 ng/ml, n = 12). By Day 62, eight of the ten remaining ewes had showed increased LH secretion. By the end of the experiment (Day 70), the mean concentration of LH in photostimulated ewes was 3.1 ± 0.2 ng/ml (n = 12), 5.4 times greater than before photostimulation (Day 0). In contrast, none of the photo-inhibited ewes showed an increase in LH secretion (Figure 27).

In contrast to LH secretion, there was no consistent increase in LHRH secretion in the photostimulated ewes (Figure 25). LHRH concentration varied from 5.5 ± 0.2 pg/ml (n = 6, Day 46) to 8.4 ± 0.6 pg/ml (n = 5, Day 67). The concentration of LHRH at the end of the experiment (Day 70) was 6.7 ± 0.6 pg/ml (n = 4). This is 1.1 times the concentration at Day 0. These two concentrations are not significantly different (Mann-Whitney *U*-test).

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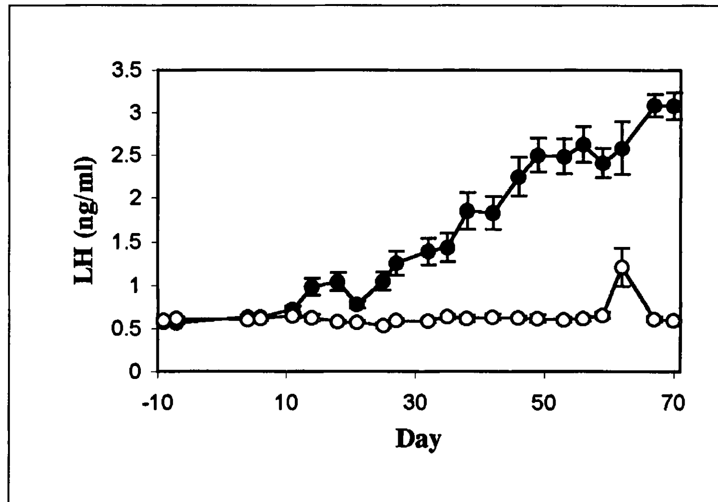

Figure 24. LH secretion (mean ± SEM) in photostimulated (closed circles) and photo-inhibited (open circles) ewes over the course of the study.

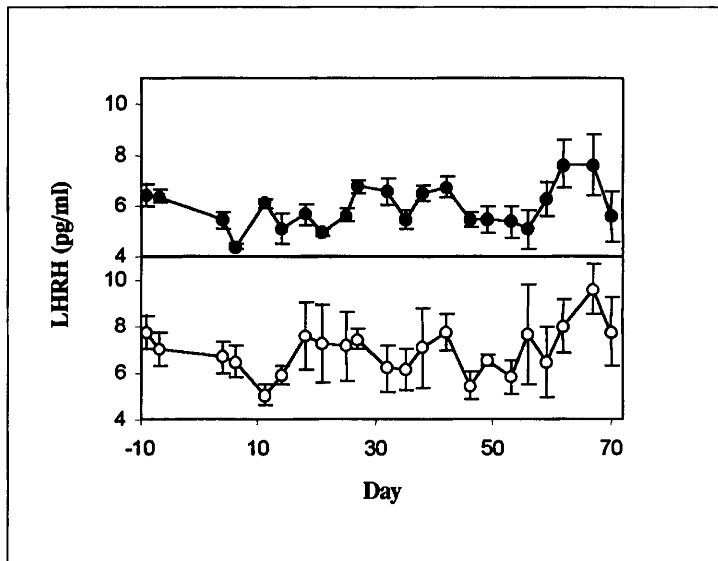

Figure 25. LHRH secretion (mean ± SEM) in photostimulated (closed circles) and photo-inhibited (open circles) ewes over the course of the study.

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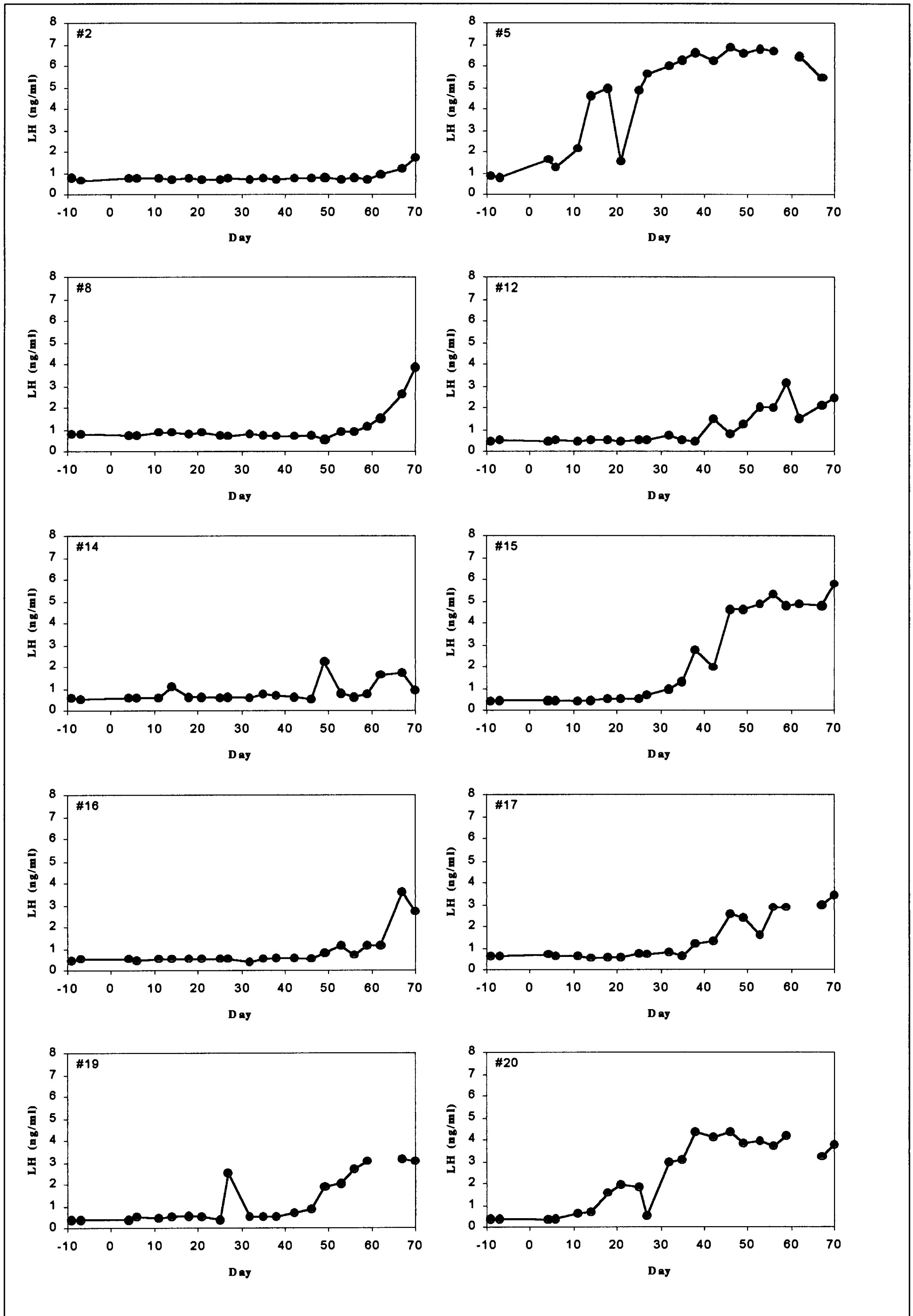

Figure 26. LH secretion in ten photostimulated ewes through the study.

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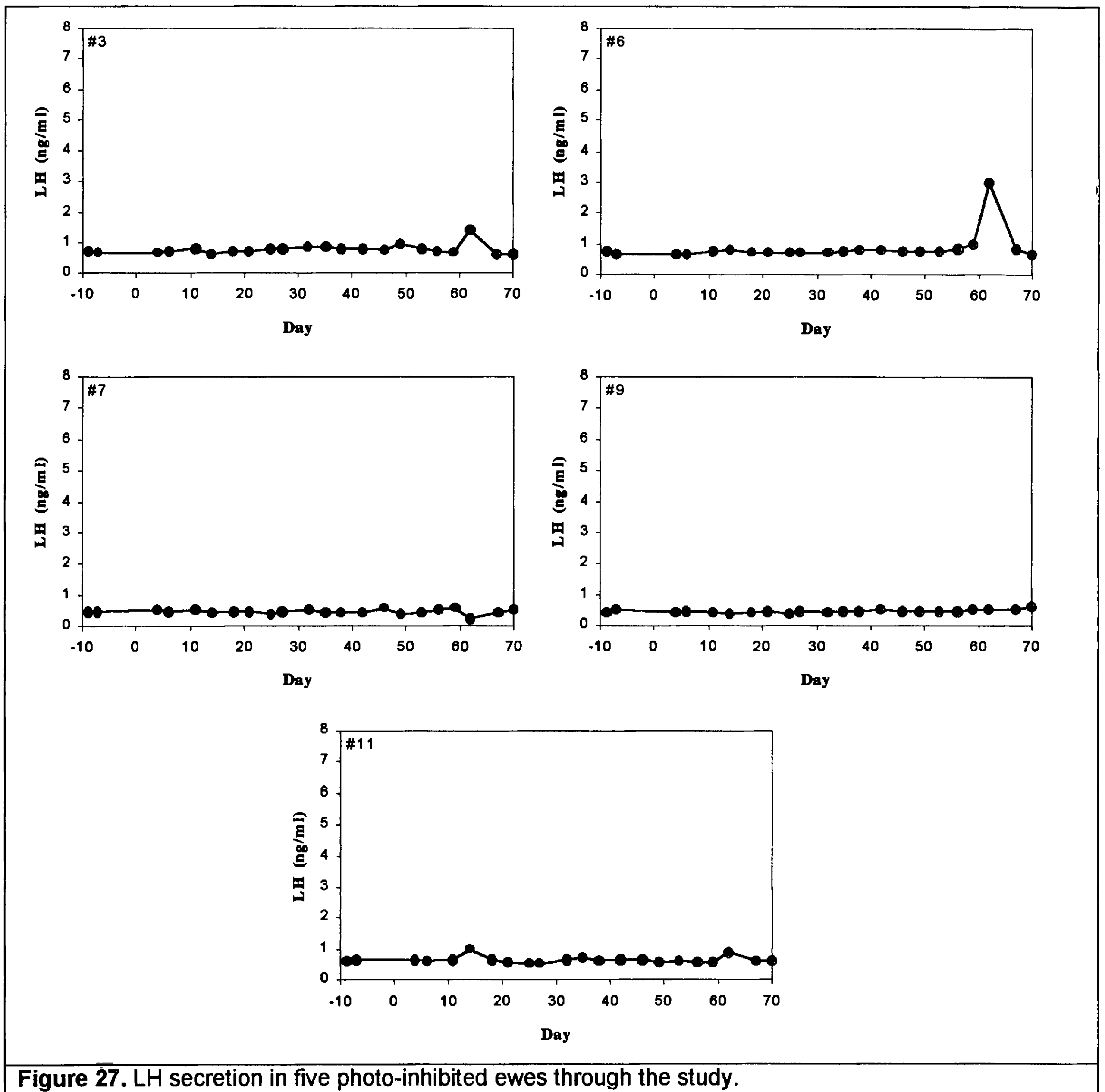

Figure 27. LH secretion in five photo-inhibited ewes through the study.

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Discussion

A number of methods have been devised for sampling LHRH and/or LH close to, or at, the locations where LHRH is produced and acts, including portal cannula and catheter systems (Fink *et al.* 1967, Porter *et al.* 1970, Fink & Jamieson 1976), focal perfusion techniques (Levine & Ramirez 1980), local microdialysis approaches (Levine & Powell 1989) and pituitary venous catheterisation (Irvine *et al.* 1986). These methods allow a direct evaluation of the regulation of LHRH secretion by factors in the internal and external environment.

In sheep, the portal-pituitary cannulation technique has provided important insights into the functioning of the LH-LHRH system. Clark & Cummins (1982) introduced the technique in sheep and were able to show for the first time the temporal association of LHRH and LH pulses. The portal-pituitary technique still involves a degree of pituitary stalk transection. Lesioning of the portal vessels can lead to reduced LH secretion due to reduction of the blood supply to the pituitary and portal blood samples can be contaminated with CSF and peripheral blood (Clark & Cummins 1982, Karsch *et al.* 1987). Perhaps the biggest drawback this and all other techniques have in common is the fact that animals cannot act as their own control in experiments and long-term experiments are impossible using the same animals. This is due to the fact that the sampling process causes necrosis of the pituitary vasculature, which results in reduced LHRH secretion as sampling progresses (Caraty *et al.* 1994). Repeated sampling is thus possible but does not provide reliable data.

The LHRH neurosecretory system lies in close proximity to the ventricular system. Specifically, the median eminence forms the base of the third ventricle with the result that LHRH diffuses into the CSF of the third ventricle. LHRH can be detected in the CSF of the entire ventricular system, and cannulation has been successfully used to determine LHRH levels in ventricular CSF (Van Vught *et al.* 1985).

The CSF method has a number of advantages over other methods. First, CSF can be collected without the aid of anaesthesia and pituitary stalk sectioning is not necessary to determine LHRH secretion. Second, the third ventricle is readily accessible for cannulation. The damage

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to the hypothalamus usually incurred by the insertion of perfusion or microdialysis probes is avoided and the placement of cannulae can be verified with X-rays, without having to kill the animals. Third, the fact that serial samples can be taken over long periods makes it possible to perform successive (long-term) studies under various endocrine conditions in the same animal.

Cannulation of the third ventricle has proved successful in assessing short-term LHRH secretion in ewes (Skinner *et al.* 1995). As this study aimed to assess long-term changes in the secretion of LHRH and neurotransmitters in ewes using the CSF sampling method, it was necessary to first determine whether this technique can be used to address long-term changes in the LHRH system in individual animals, something which has thus far proved elusive using other techniques. Accordingly, the effect of chronic and acute CSF-sampling on the functioning if the LH-LHRH system, as well as the neurotransmitters (amino acid and catecholaminergic) that affect the functioning of this system, was examined in an experiment that was designed to evaluate the CSF method and, at the same time, gather data to address long-term changes in LHRH and neurotransmitter secretion.

Effect of CSF sampling on neurosecretion

There were no significant differences in concentrations of LH and LHRH between the CSF-sampled and unsampled animals. This suggests that sampling did not fundamentally affect the LH-LHRH neurosecretory system. However, this should be viewed with caution as the small sample sizes precluded any meaningful statistical analysis of the data.

Asp, Glu and GABA secretion decreased in both the CSF-sampled and unsampled groups, although the unsampled animals were slightly more marked in their response. Similarly, although the entire picture is not clear (due to a loss of data for the low pulse situation), both groups had very similar concentrations of DOPAC, 5-HIAA and 5-HT after photostimulation.

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In the ewes in this study, LH secretion increased after photostimulation. This is in agreement with previous studies in which short days stimulated LH secretion (Bittman *et al.* 1985, Viguié *et al.* 1995*a*). LH secretion in the photostimulated group increased steadily over the course of the study. While this might suggest that the stimulation of LH secretion was itself gradual, this is largely an artifact of the "early" increases in ewes 5 and 20, which masks the more dramatic increases observed in the remaining animals at later stages of the study. Thus, the latency in the increase of LH secretion observed by Viguié *et al.* (1995*a*) was corroborated in this study.

The increase in LH secretion that is normally observed after photostimulation is due to increased LHRH secretion (Viguié *et al.* 1995*a*). However, the expected increase in LHRH secretion did not occur in photostimulated ewes in this study. The mean concentration of LHRH was not significantly different between the groups. The withdrawal of CSF from the third ventricle could have had an effect on the concentration of LHRH measured in the sampled CSF and/or could have affected the functioning of the LHRH system itself. The former possibility is more likely, as any direct effect on LHRH secretion would have been translated into decreased LH secretion, which is clearly not the case.

In addition to altering LHRH neurotransmission directly, sampling may have affected the secretion of neurotransmitters that modulate LHRH secretion. Thus, the decrease in LHRH secretion in the photostimulated ewes may be due to decreased secretion of the excitatory amino acids, Asp and Glu. While both Asp and Glu levels in the photostimulated ewes decreased, only Glu decreased in the photo-inhibited ewes; Asp secretion increased in these animals. This difference in Asp secretion between the photo-inhibited and photostimulated ewes suggests that the discrepancy in LHRH secretion between the groups could be due to Asp secretion. Thus, while Glu might stimulate (or support) LHRH secretion in anoestrus, Asp could be the dominant supportive amino acid in oestrus, i.e. an increase in Asp secretion might counteract a depression in LHRH secretion as Glu secretion decreases. This is supported by the finding that the response of ewes to NMDA, a glutamatergic agonist, is greater in long days than in short days (Viguié *et al.* 1995*b*). Indeed, this would account for the

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fact that LHRH secretion increased slightly in the photo-inhibited ewes in association with increased Asp secretion, while the decreased secretion of Asp in the photostimulated ewes may be responsible for the observed decrease in LHRH secretion. The fact that the photo-inhibited ewes showed an increase in LHRH secretion (however slight) could also be due to the persistence of an endogenous rhythm or their photoperiodic history (Woodfill *et al.* 1994).

Apart from the intergroup difference in Asp secretion, the groups showed no differences with respect to the other neurotransmitters measured. Thus, GABA and DOPAC secretion decreased in both photostimulated and photo-inhibited ewes. This might have been expected in the ewes that were photostimulated as they moved from anoestrus into oestrus. Both the GABAergic and dopaminergic systems mediate negative feedback on LHRH secretion by oestradiol (Demling *et al.* 1985, Havern *et al.* 1994). Thus, a decrease in GABA and dopamine secretion would alleviate the negative feedback of oestradiol and facilitate the stimulation of LH and LHRH secretion. However, the expected increase in LHRH secretion did not occur in the photostimulated ewes. This suggests that GABA and dopamine have a mediatory role in LHRH secretion. Thus, while GABA and dopamine suppress LHRH secretion in ewes with low LH levels, the decrease in GABA and dopamine secretion that occurs with the transition to oestrus need not necessarily lead to an increase in LHRH secretion. Rather, stimulation of LHRH secretion might require an increase in the secretion of other, stimulatory neurotransmitters, such as excitatory amino acids. Indeed, the fact that Asp secretion increased in the one group that showed an increase in LHRH secretion, but not in the other group (in which LHRH secretion did not increase), lends support to this hypothesis.

5-HT secretion increased in both groups over the course of the study, as indicated by the increased levels of this neurotransmitter and its chief metabolite, 5-HIAA. 5-HT generally inhibits gonadotrophin secretion in ewes during anoestrus (Li *et al.* 1995). Thus, the fact that LH secretion in photostimulated ewes increased, despite increased 5-HT levels, suggests that 5-HT might only be effective in inhibiting LH secretion in anoestrus, when it amplifies the negative feedback of oestradiol. Alternatively, the increase in 5-HT may have been responsible for the observed decrease in LHRH secretion after photostimulation, which would suggest that it acts on the LHRH system without affecting LH secretion. However, this is unlikely to be the

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case because first, 5-HT may regulate the secretion of hormones other than LHRH and second, the decrease in LHRH may be due to the disruption of the normal functioning of the LHRH neuronal system.

Conclusion

In conclusion, the present study has confirmed the practicability of the CSF method for addressing the LH-LHRH neurosecretory system. More importantly, the study has showed that the CSF method is suitable for addressing long-term experimental questions regarding the functioning of this system and there is potential to expand the technique to address the neural system that underlies LHRH neurosecretion.

These conclusions are encouraging but need to be put into perspective by the problems encountered. First, while mortality was not great—particularly when compared to other studies—the number of animals in which CSF stopped flowing needs to be reduced. Secondly, the apparent effect of CSF-sampling on the neuroendocrine axis needs to be investigated more thoroughly. Certainly, improvements in this relatively new technique should resolve these problems in the near future. Finally, it is important to note that the concentration of substances measured in CSF represents a pool of molecules derived from secretory structures located in different parts of the brain that have different physiological functions. In contrast, techniques such as microdialysis target specific areas of the brain and are able to measure the secretory products of such areas with a high degree of specificity. Thus, CSF measurements need to be viewed with this in mind and have to be analysed with appropriate controls and interpreted carefully.

As a result of the problems encountered with the CSF sampling method, the data should be interpreted with caution. Furthermore, the fact that sample sizes were frequently too small to perform meaningful statistical analyses means that the conclusions reported here are tentative. Despite this, however, the data suggest that neurotransmitters may have differential roles that are determined by the reproductive state of an animal. For example, a neurotransmitter may inhibit LHRH secretion in the anoestrous season, but have no discernable role in oestrus.

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Furthermore, the dimunition in secretion of a neurotransmitter (e.g. an inhibitory substance) does not necessarily mean that the neuroendocrine axis will exhibit the opposite effect (i.e. stimulation). Thus, these observations support the contention that there is a complex system of interneurones that link melatonin to the modulation of gonadotrophin secretion. The form of the increase in gonadotrophin secretion suggests that in sheep this neuromodulatory system requires several weeks to finally exert its effects on the secretion of gonadotrophins. Thus, there is likely to be a hierarchy of neurotransmitters that interact with other neural components to modulate LH and/or LHRH secretion.

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Chapter 7

Synthesis

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In the first major theme of this thesis (Chapter 3, 4 & 5), I addressed the phenomenon of aseasonal reproduction in ungulates. This was motivated by the fact that there is a dearth of knowledge regarding the regulation of reproduction in aseasonally breeding ungulates. This is in contrast to seasonally breeding ungulates, which have been extensively studied. As the physiological mechanisms underlying seasonal reproduction are relatively well established, I sought to examine an aseasonal ungulate to determine whether or not the basic physiological system that underpins seasonal reproduction exists in aseasonal ungulates. The model I selected was the putatively aseasonal ungulate, the springbok (*Antidorcas marsupialis*). My first goal was to determine whether or not reproduction in springbok is regulated according to environmental variables. I discovered that springbok do not use photoperiod or other climatic *zeitgebers*, such as temperature and rainfall, to cue reproduction (Chapter 3). However, this conclusion was largely based on data from wild springbok populations, where it is impossible to assess one factor, such as photoperiodism, without controlling for the effect(s) of external factors, such as nutrition and predation. Accordingly, I next investigated whether the reproductive characteristics of a captive herd of springbok, held in controlled conditions, are consistent with those of seasonal species that employ cues such as photoperiod to regulate reproduction.

The results presented in Chapter 4 suggest that springbok do not conform to the characteristics of a typical seasonal breeder. Thus, springbok do not seem to employ photoperiod, temperature or rainfall as cues to time the onset of the reproductive cycle. However, the fact that the data were suggestive of a limited seasonal response indicates that springbok, while not strictly seasonal, may not be entirely aseasonal.

One reason for a lack of seasonality might be that the physiological mechanism that is used to interpret and use photoperiodic information might be absent. Thus, I sought to determine whether the apparent lack of strict seasonality in springbok is due to the lack of a functional pineal-melatonin system or whether springbok receive and process photoperiodic information in a normal manner, but fail to act upon it. My results show that the pineal-melatonin system of springbok is present and functions in the same way as in photoperiodic species (Chapter 5).

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Thus, the fact that springbok are not strictly seasonal breeders suggests that this species "ignores" photoperiodic information that could be used to cue reproduction. The physiological basis for the lack of seasonality is thus suggested to lie downstream from the pineal-melatonin system.

My next goal was to investigate the transition from reproductive quiescence to activity in female mammals. Unfortunately, it is not possible to address this question in springbok because there is a lack of technical facilities and expertise and springbok are not domesticated and are averse to intensive handling. For these reasons, I based the second major theme of my thesis (Chapter 6) on an alternative model, namely the sheep (*Ovis aries*). The reproductive physiology of sheep has been thoroughly investigated, particularly the relationship between photoperiodicity and reproduction. As the neuroendocrine axis that underlies reproduction is fundamentally the same in all ungulates and the pineal-melatonin system in springbok is intact, it is reasonable to assume that the photoperiodically modulated changes in the reproductive axis that occur in females coming into estrus are similar in sheep and springbok.

To address this aspect, access to the LHRH system and its neural correlates, is required. A novel method of addressing the LHRH neurosecretory system via the third ventricle has been recently developed in sheep, which offers great potential for addressing the LHRH system and its neural correlates simultaneously in the long term. However, as this method is a novel one, it was first necessary to determine whether sampling ventricular CSF is a suitable means of addressing the long-term dynamics of the LH-LHRH neurosecretory system, as well as the neurosecretory components implicated in the regulation of this system. Thus, I carried out an experiment that was designed to simultaneously assess the practicability of the CSF sampling method and collect data that could be used to address the LHRH neurosecretory system. Unfortunately, the method did not prove to be as successful as was hoped. Consequently, the data that were gathered had to be viewed with extreme caution and any conclusions drawn from them are, at best, tenuous. Nevertheless, the data did show that neurotransmitters may have differential roles that are determined by the reproductive state of an animal. For example, a neurotransmitter may inhibit LHRH secretion in the anoestrous season, but have no discernable role in oestrus. Furthermore, the dimunition in secretion of a neurotransmitter (e.g.

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an inhibitory substance) does not necessarily mean that the neuroendocrine axis will exhibit the opposite effect (i.e. stimulation). These observations support the contention that there is a complex system of interneurones that link melatonin to the modulation of gonadotrophin secretion.

In conclusion, although the physiological system that regulates reproduction in ungulates is undoubtedly complex, it nevertheless seems to be fundamentally similar in seasonal and aseasonal species. Thus, it seems that aseasonal animals may not be physiologically bound to reproduce aseasonally, but reproduce in the manner that they do because it is an adaptive advantage. In Appendix 3, I demonstrated that the existence of adaptations to time reproduction, such as the pineal-melatonin system, are not a prerequisite for the development of seasonal reproduction. On the other hand, the absence of such adaptations does not necessarily preclude an organism from reproducing seasonally. This is exemplified by the springbok, which possesses an intact pineal-melatonin system but apparently has no need for this in its natural habitat. This could be applicable to a broader range of taxa that share similar characteristics, i.e. an intact pineal system but aseasonal reproduction.

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Appendix 1

The effect of xylazine on the dynamics of luteinizing hormone secretion in ewes

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Introduction

Xylazine is widely used for sedation, mild to moderate analgesia and muscle relaxation to facilitate the performance of medical examinations, treatment and surgical procedures (Booth 1982). Xylazine is an alpha-2 adrenoreceptor agonist (Kobinger 1978) that binds to alpha-2 adrenoreceptors of spinal and supraspinal noradrenergic neurones (Goodchild *et al.* 1996). Centrally administered xylazine induces hypotension, bradycardia, heart block, respiratory depression and hyperglycemia (Booth 1982) while epidural administration of xylazine fails to affect these parameters (Greene *et al.* 1995) and intrathecal administration increases the threshold for nociception in some peripheral sites (Goodchild *et al.* 1996). The primary site(s) of action are thus suggested to lie in the brain.

The effects of NA on LH and LHRH secretion are well documented and were reviewed in Chapter 1. NA released by noradrenergic neurones in the median eminence of the brain inhibits LHRH secretion in ewes (Robinson *et al.* 1991, Goodman *et al.* 1993). LHRH is synthesised in the LHRH neurone cell bodies that are located in the preoptic area of the hypothalamus and is released from the nerve terminals in the median eminence into the portal blood vessels of the hypophysis. LHRH acts on gonadotrophic cells in the neurohypophysis to effect the synthesis and release of LH. NA thus indirectly affects LH secretion through LHRH secretion.

Considering the above, it is probable that any substance that alters NA secretion might also affect LH secretion (Barraclough 1995). However, the effect of xylazine—arguably the most ubiquitous of alpha-2 adrenoreceptor agonists—on neurohormone secretion has not been addressed. The nature of the experiments presented in Chapter 4 and 5 require intensive handling of the animals and chemical restraint was considered. Although xylazine is regularly used as a means of pharmacological restraint in physiological studies of wild animals (e.g. Marais *et al.* 1991), the effect of xylazine on the production and secretion of hormones and their metabolites has not been satisfactorily addressed. Thus, my aim was to determine whether xylazine can be used as a means of chemical restraint without affecting centrally mediated neurohormone (specifically, LH) secretion.

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Blood samples were taken at 15 min intervals for 6 hours. The collection was timed to correspond to the subjective late afternoon to avoid any possibility of early morning feeding affecting LH secretion.

After 3 hours of sampling, xylazine HCl (0.01 ml/kg, Rompun®, Bayer) was administered by rapid intravenous injection into each animal in group 1 immediately after blood had been collected from its jugular vein. Animals in group 2 did not receive xylazine.

LH radioimmunoassay

LH was assayed in duplicate 100 μl aliquots of plasma using the radioimmunassay method of Pelletier *et al.* (1968), modified by Montgomery *et al.* (1985). All samples were analysed in a single assay, for which the intra-assay CV for 5 plasma pools averaged 6.5 %. Sensitivity was 0.13 ng/ml of 1051-CY-LH.

Analysis of data

The following secretion parameters were analysed: mean concentration of LH secreted, mean number of LH pulses and the mean pulse amplitude. Pulse peaks were detected using the Munro algorithm calibrated as follows. Rise threshold = 2.00; Baxter parameters $B1$, $B2$ and $B3$ = 0.21205, 0.025 and 0.00039, respectively; G parameters $G1$, $G2$, $G3$, $G4$, and $G5$ = 3.980, 2.400, 1.680, 1.240 and 0, respectively.

To compare the two groups, a Mann-Whitney *U*-test was performed using the differences (defined as the value after treatment minus the value before treatment) in response between the two periods for each animal, for each secretion parameter.

Results

The difference in the concentration of LH secreted in each period was significantly different between the groups (Mann-Whitney *U*-test; $z = -2.45$; $p = 0.01$), the difference being greatest

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in group 1 (Table 5). Similarly, the difference in the concentration of LH secreted with each pulse (equivalent to the pulse amplitude) differed significantly between groups (Mann-Whitney *U*-test; $z = -2.04$; $p = 0.04$) and was greatest in group 1 (Table 5). The difference in the number of pulses after treatment did not differ significantly between groups (Mann-Whitney *U*-test; $z = -1.95$; $p = 0.05$). However, the number of pulses in group 1 decreased threefold after xylazine administration, from 0.63 ± 0.08 to 0.21 ± 0.09 pulses/hr while the number of pulses in group 2 increased from 0.33 ± 0.00 to 0.56 ± 0.22 pulses/hr (Table 5). It should be emphasised that the lack of significance in this instance may be due to the small sample size and should thus be interpreted with caution.

Discussion

Previous studies have suggested that the noradrenergic system may play a role in the control of LH secretion (Meyer & Goodman 1985, Meyer & Goodman 1986, Havern *et al.* 1991). Apparent "pulses" of NA have been reported in the median eminence of a number of mammalian species, including rats (Jarry *et al.* 1990), monkeys (*Macaca mulata*, Terasawa *et al.* 1988) and sheep (Robinson *et al.* 1991). While it has been proposed that these pulses might actually drive LHRH secretion in primates (Terasawa *et al.* 1988), there is little evidence to support this in ewes (Robinson & Kendrick 1992). Nevertheless, there seems little doubt that NA does play a role in the modulation of LH-LHRH secretion in ewes (Goodman *et al.* 1993).

It is reasonable to expect any substance that affects NA secretion to also affect LH secretion, albeit indirectly, because oestradiol suppresses LH secretion in ewes as a consequence of decreasing mean NA levels (Havern *et al.* 1991). Indeed, a number of studies have shown that various adrenoreceptor agonists and antagonists affect the amplitude and/or frequency of LH pulses (Barraclough 1995, Goodman *et al.* 1995). The results of the experiment presented here provide additional support for this contention, as both the pulse amplitude and frequency of LH secretion was dramatically depressed after the administration of the adrenoreceptor agonist xylazine. Evidence that Thus, xylazine probably achieves its effects by increasing the synthesis and release of NA, which in turn acutely suppresses LHRH and subsequently LH secretion.

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In conclusion, the results presented here show that xylazine disrupts the normal functioning of the LH-LHRH neurosecretory system. Consequently, adrenoreceptor agonists such as xylazine should not be used as a means of pharmacologically restraining animals in studies that require an accurate assessment of hormone levels. The use of xylazine to effect sedation and facilitate the handling of the springbok used in this study was thus not pursued.

Table 5. The effect of xylazine on parameters of LH secretion (mean ± SEM) before and after xylazine injection. The value of *p* refer to differences between group 1 and group 2.

Parameter	Group 1		Group 2		*p*
	Before	**After**	**Before**	**After**	
Mean LH concentration	3.64 ± 0.36	3.09 ± 0.33	3.29 ± 0.11	3.69 ± 0.22	< 0.05
Number of LH pulses/hr	0.63 ± 0.08	0.21 ± 0.09	0.33 ± 0.00	0.56 ± 0.22	0.05
Mean pulse concentration	4.79 ± 0.28	4.37 ± 0.63	4.06 ± 0.07	4.64 ± 0.18	< 0.05

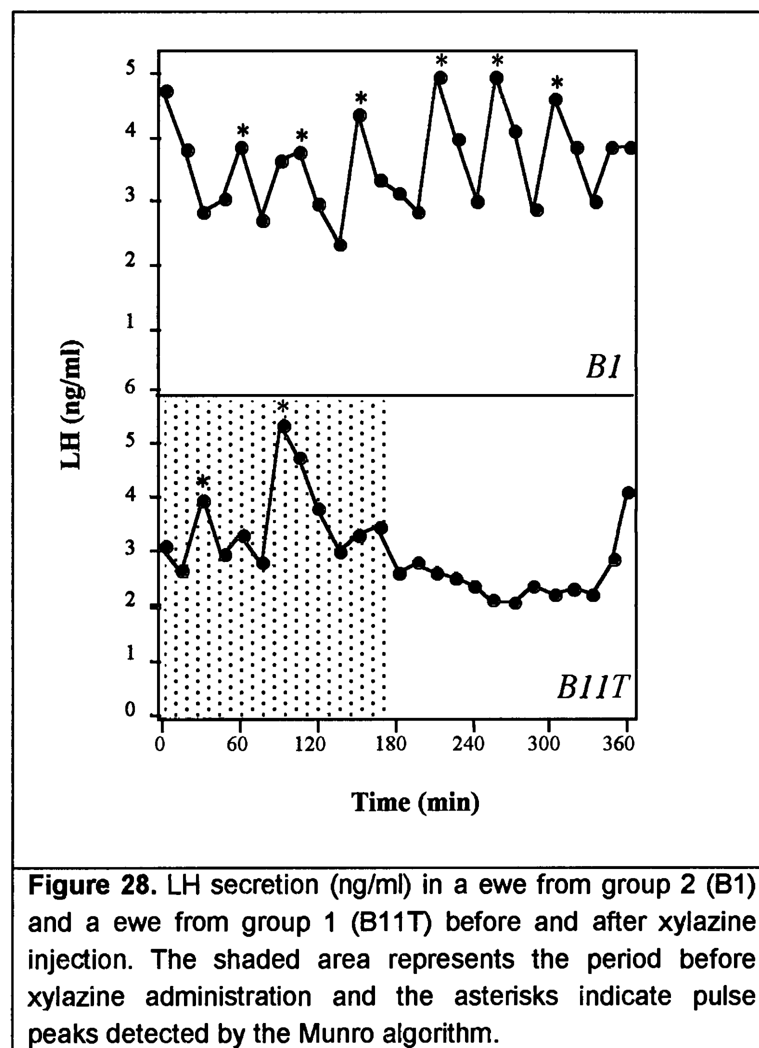

Figure 28. LH secretion (ng/ml) in a ewe from group 2 (B1) and a ewe from group 1 (B11T) before and after xylazine injection. The shaded area represents the period before xylazine administration and the asterisks indicate pulse peaks detected by the Munro algorithm.

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Appendix 2

The index of restriction

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Introduction

The most favourable time for an animal to give birth depends on each of the variables that affect its own survival and the survival of its offspring. In ungulates, the main factors that affect survival and hence reproductive success are nutrition and predation (Rutberg 1987). Thus, ungulates in the temperate regions of the northern hemisphere restrict calving to the warm spring and summer months to avoid harsh winter conditions (Grubb & Jewel 1973); in tropical climes, ungulates may restrict calving to flood predators (Estes 1976, Estes & Estes 1979). In some instances, calving is restricted for both reasons (e.g. *B. bison*, Rutberg 1984).

Spinage (1973) defined seasonal breeding as "...the confinement of matings and births to a restricted part of the year". Restricted breeding has been defined as "...the non-random or seasonal bearing of offspring" (Skinner & Van Jaarsveld 1987) and "...the birth of offspring within a short time frame" (Berger 1991). According to these definitions, the terms *seasonal* and *restricted* are, in fact, synonymous. For example, the blesbok (*Damaliscus dorcas phillipsi*) is seen as a highly seasonal breeder by Skinner *et al.* (1974) and Spinage (1973) classified the wildebeest (*C. taurinus*) as a restricted breeder. Yet, despite this distinction, there is very little difference between the calving patterns of either species (Figure 29).

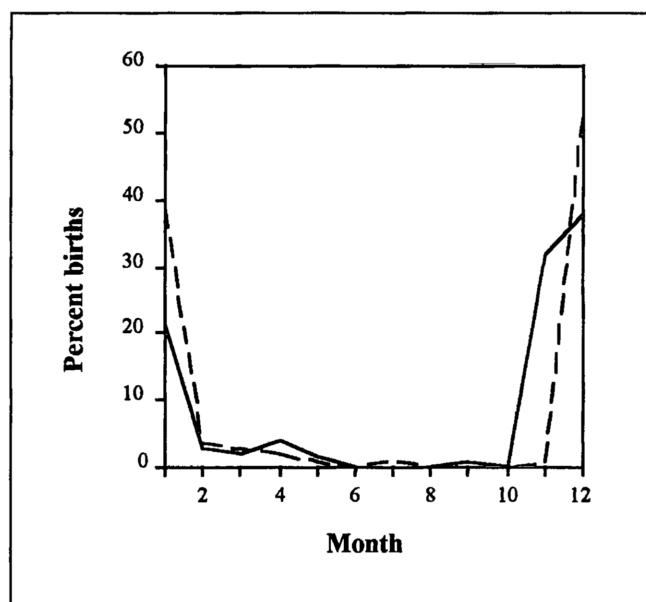

Figure 29. Annual distribution of births in a wild population of blesbok (*Damaliscus dorcas phillipsi*) and blue wildebeest (*Connochaetes taurinus*) from the same southern African locality. Data from Skinner *et al.* (1974).

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Most species attempt to limit their reproduction to the most favourable period of the year. Assuming that they are able to control the timing of reproduction, births will be regular if fluctuations in environmental conditions are regular; this is essentially what is implied by seasonal breeding. However, the vagueness of the definitions presented above highlights the lack of definitive, objective criteria by which species can be classified as either restricted breeders or not. Some species that are neither obviously seasonal nor obviously aseasonal are classified as seasonal by some authors and aseasonal by others. By what criteria can the distinction between seasonal and aseasonal breeding be made?

The index of restriction

I suggest that the classification of species as either restricted breeders or not, is unhelpful; rather, the degree of restriction should be viewed as a continuum ranging from completely non-restricted to absolutely restricted, and should be quantified as such. Thus, if we take the mean monthly proportion of births in a year, the theoretical non-restricted limit to the continuum would be a species with exactly the same proportion of births in every month of the year (Figure 30*a*). On the other hand, the ultimate in restriction would be a species where all births occur in a single month and no births occur in any other month (Figure 30*c*).

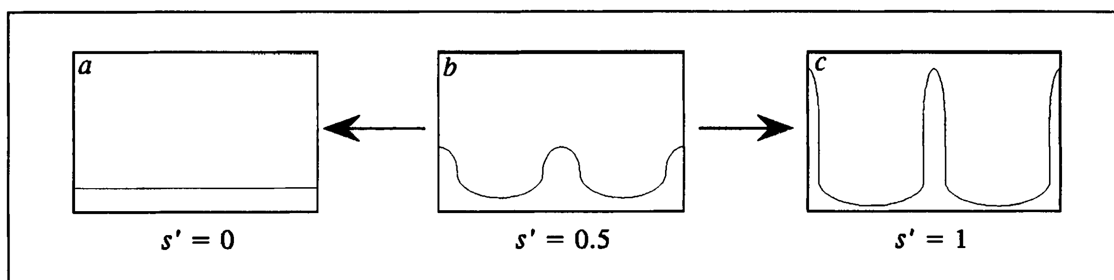

Figure 30. Schematic representation of the continuum of restriction in calving. Each box represents the theoretical relative frequency (*y*-axis) distribution of births for a single year for (*a*) an unrestricted ($s' = 0$), (*b*) moderately restricted ($s' = 0.5$) and (*c*) highly restricted ($s' = 1$) hypothetical population . The favourable period of the year is that area of the box in which the proportion of births increases in box (*b*) and (*c*).

Having thus defined the limits of the continuum, what is needed is a quantitative measure or index of the degree to which calving is restricted. A suitable index should quantify the degree of "skew" in the distribution of births, which can be assumed to equate to the degree of

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restriction. For instance, a highly skewed distribution is the result of births being restricted to a few months only (Figures 30 & 31).

There are a number of methods that can be used to quantify skew. Rutberg (1987) calculated the number of consecutive days during which 80% of the annual births occurred and used this as a measure of restriction. However, without data in the appropriate form, it is either difficult or impossible to apply this method. Distribution fitting is another option, but is not suitable because it cannot resolve the calving pattern sufficiently. There are a number of mathematical measures that describe the dispersion of data about a mean value, including the range, interquartile range, standard deviation, variance and standard error (Samuels 1994). However, the measure that best suits the purpose of describing the skew in proportional distribution of births is the standard deviation, s. (Variance and standard error are simply transformed measures of s.) In contrast to the range and interquartile range, s takes all data into account. Furthermore, as s is unaffected by linear data transformations, any raw data set can be transformed into relative proportions.

To facilitate its interpretation, the index should have a range of 0 to 1. We can achieve this by simply expressing any value of s as a fraction of the maximal possible value of s to give a relative value; this is effectively the index, s'. The index of restriction can thus be defined as:

$$s' = \frac{s}{s_{max}} \qquad \text{where} \qquad 0 \leq s' \leq 1$$

s_{max} refers to the maximal value of s for a particular frequency distribution. The frequency distribution employed here is the relative monthly proportion of births calculated for each month of the year (i.e. for 12 months). Data that are not in this form can be easily modified. The range of values of s for data in this form is from 0 (when the proportion of births per month is exactly equal) to 0.289 (when all births occur in a single month). The index value is thus calculated as $s/0.289$.

s' can be readily used to quantify the extent to which calving is restricted in any species for which birth data are available. Furthermore, s' is dimensionless and easily interpretable: high

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values indicate highly restricted species and vice versa. Furthermore, the index is unaffected if it is calculated from different frequency distributions.

Rutberg's hypothesis: does phylogeny determine calving patterns in ungulates?

There is evidence that phylogeny may affect the degree of restriction in ungulates. For example, many Alcelaphines (e.g. wildebeest; blesbok; bontebok, *D. d. dorcas*; tsessebe, *D. lunatus lunatus*) have relatively highly restricted calving periods, despite occurring in vastly different environments (Du Plessis 1972, Child *et al.* 1972, David 1973, 1975, Estes 1976). However, the variation in restriction within species and individuals prompted Rutberg (1987) to suggest that the lengths of ungulate birth seasons are not constrained by phylogeny. For instance, impala (*Aepyceros melampus*), sable (*Hippotragus niger*) and Coke's hartebeest (*Alcelaphus buselaphus*) breed throughout the year in equatorial East Africa but have discrete breeding seasons in southern Africa (Kayanja 1969, Leuthold 1970, Spinage 1973, Murray 1982). While this is an attractive verbal argument, an empirical test of Rutberg's hypothesis can easily be made using the quantitative index of restriction, *s'*, as defined above.

Thus, a solution to the question of the effect of phylogeny on restriction might be provided by contrasting the degree of restriction in wild and captive populations of the same species. Wild populations show a significant increase in restriction with increasing latitude north and south of the equator Spinage (1973). A number of factors, including predation, rainfall (i.e. nutrition), temperature and phylogeny may determine the degree of restriction. Constraints such as nutrition, predation and temperature that are commonplace in the wild are, at best, absent or, at worst,

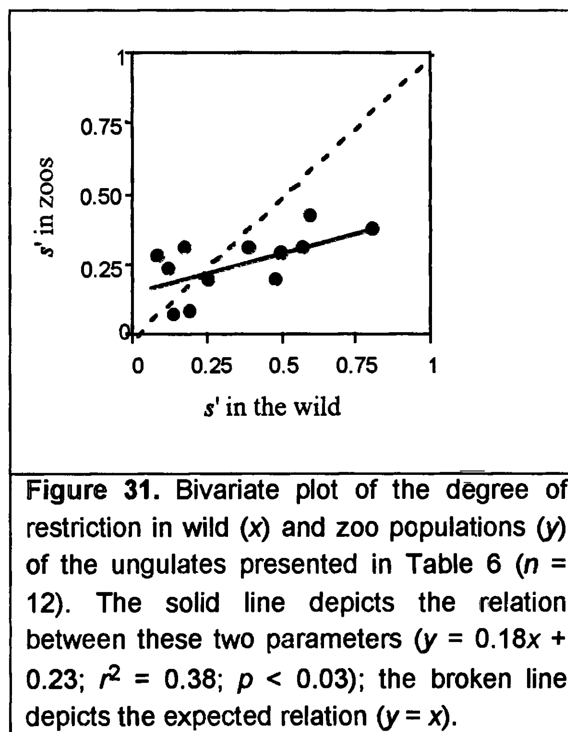

Figure 31. Bivariate plot of the degree of restriction in wild (x) and zoo populations (y) of the ungulates presented in Table 6 (n = 12). The solid line depicts the relation between these two parameters ($y = 0.18x + 0.23$; $r^2 = 0.38$; $p < 0.03$); the broken line depicts the expected relation ($y = x$).

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constant in captivity. Thus, if phylogeny constrains restriction, the relationship between restriction and latitude observed in wild populations should also hold in zoo populations where the possibly confounding effects of other factors are more controlled. Thus, we should expect there to be a uniform, linear relation between restriction in zoo and wild populations of the same species.

Indeed, this appears to be the case: the slope of the relation between restriction in zoo and wild populations of the same species is not significantly different to the slope of the predicted relationship (ANCOVA, $F = 0.58$, $p = 0.46$, $n = 12$). However, the fact that the slope of the observed relation is lower than predicted (Figure 31) indicates that the zoo populations are generally less restricted than their wild conspecifics, although the average degree of restriction in the zoo populations is not significantly lower than wild populations (Wilcoxon matched pairs test, $Z = 2.37$, $p = 0.02$, $n = 7$). Thus, there appears to be a modest, but statistically insignificant, decrease in restriction in response to the constant nutritional conditions and absence of predators. Indeed, a similar analysis of the breeding records of 45 ungulate species maintained in London Zoo revealed a significant correlation between restriction and the latitude of their natural habitat (see Figure 9, p. 32). This suggests that the degree to which reproduction is restricted, at least in ungulates, must be phylogenetically determined to lie within certain moderately flexible limits. While the influence of nutrition and predation are controlled for in zoo populations, captive and wild populations in the same latitudes are generally exposed to similar temperatures. Presumably, if temperature were controlled in captive ungulate populations, the difference in terms of restriction between these and wild populations would be exacerbated. In conclusion, the balance of the preceding empirical evidence does not support Rutberg's hypothesis: phylogeny does constrain restriction of the calving period in ungulates.

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Table 6. Summary of the index of restriction in wild and captive (zoo) populations of 12 ungulate species. *London Zoological Gardens (51°30'N 0°5'W); source: Zuckerman (1953). †National Zoological Gardens of Pretoria (25°45'S 28°10'E); source: J.D. Skinner, personal communication.

Species	Wild	Zoo	Source
B. bison	0.598	0.429*	Rutberg 1984
Cervus elaphus	0.804	0.382*	Guinness *et al.* 1971
Giraffa camelopardalis	0.126	0.245*	Hall-Martin 1975
Equus hartmannae	0.265	0.201†	Joubert 1971
Kobus leche	0.501	0.294*	Spinage 1973
Oryx gazella gazella	0.200	0.093†	Dieckmann 1980
Syncerus caffer	0.399	0.311*	Sinclair 1974
Taurotragus angasi	0.150	0.076†	Anderson 1978
Taurotragus oryx	0.482	0.202*	Buys 1987
Taurotragus strepciceros	0.572	0.320†	Allen-Rowlandson 1980
Tragelaphus scriptus	0.188	0.315*	Allsopp 1971
Kobus ellipsiprymnus	0.095	0.292†	Spinage 1982

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Appendix 3

The development and maintenance of birth distributions

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Introduction

The annual birth distributions of most animals show some degree of skew with a greater proportion of births being restricted to that period of the year that is most propitious to survival of the mother and offspring (Sadlier 1969). Skew is thus assumed to be the result of individuals taking advantage of the most favourable environmental conditions (Leuthold & Leuthold 1975). Since individual fitness depends on the number of surviving offspring produced, natural selection presumably acts on populations of animals to produce skew under the appropriate conditions, i.e. under conditions where the fitness of individuals and their offspring is greater in one period of the year than at another time. The degree of skew is analogous to the degree to which reproduction is restricted (see Appendix 2, p. 90). On a gross scale, the degree of restriction depends on the degree to which the environment fluctuates seasonally (see Appendix 2, Rutberg 1987). An index of restriction was developed in Appendix 2 that facilitates the standardisation and quantification of reproductive skew. In this Appendix, I develop a model that describes how skewed birth patterns are developed and sustained and examine the effect of variability in the duration of favourable seasonal conditions and mortality due to environmental pressure, on reproductive skew. In addition, I examine the effect of obligate and facultative seasonal reproductive adaptations on reproductive skew.

The model

I assume that during a single year there are two contrasting periods or seasons, one more favourable than the other. The nature of a season can depend on anything from meteorological conditions (e.g. warm and cold weather) or nutritional constraints to the presence/absence of predators. However, to illustrate this model, I define the favourable period as being a rainy (wet) season and the unfavourable period as a dry season.

Demography

Let the probability of mortality of neonates born in the wet season be μ and in the dry season, β. The probability of mortality is here defined as the likelihood of an animal dying due to the

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combined effect of biotic factors (i.e. predation, disease and intra- and interspecific competition) and abiotic factors (i.e. climatic conditions). Together, these factors are henceforth referred to as environmental pressure. I assume that survival is greater in the wet season, i.e. $\mu \leq \beta$. At time t, the number of neonates (nn) in each season is directly proportional to the number of sexually mature females that give birth (N), assuming that all such females in a population give birth. I assume that offspring reach puberty after one year and are mated after that time. The number of neonates that survive their first year (from time $t - 2$ to time $t - 1$) to reach puberty and be mated in the wet season is

$$nn_{wt-1} = N_{wt-2}(1 - \mu) \quad [1]$$

and in the dry season

$$nn_{dt-1} = N_{dt-2}(1 - \beta) \quad [2]$$

The number of neonates that survive two years (from time $t - 2$ to time t) to give birth to their own offspring in the wet season at time t is

$$nn_{wt} = N_{wt-2}(1 - \mu)^2 \quad [3]$$

and in the dry season

$$nn_{dt} = N_{dt-2}(1 - \beta)^2 \quad [4]$$

If the probability of mortality for adult females in the wet season is ϕ and ε for the dry season, the number of offspring born to adult females (n) in the wet season is

$$n_{wt} = N_{wt-1}(1 - \phi) \quad [5]$$

and in the dry season

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$$n_{dt} = N_{dt-1}(1-\varepsilon)$$ [6]

Variability in the duration of the favourable season

The number of births in each season depends on the number of females that were mated at such a time as to give birth in that season. Assuming a gestation length of 6 months for a medium sized ungulate (e.g. springbok, Skinner & Louw 1996) and a duration of 6 months for both the dry and wet season, a female mated in one season will give birth at a coincident time (i.e. 6 months later) in the opposite season. For example, if a female is mated in the fifth month of the dry season, she will give birth in the fifth month of the wet season. However, if the duration of a season is not fixed, its occurrence cannot be predicted accurately. Thus, some births that are the result of matings in the dry season may occur in the dry season instead of the wet season, and vice versa. The probability of this occurring depends on the length of each season and how variable the duration of a season is. If t represents one year then the duration of the wet season at time t is r_t, where r_t is a proportion of the year ($0 < r_t < 1$). The number of offspring born to females that give birth in the "wrong" season (i.e. in the same season as in which they are mated) is then

$$n_{wt} = \begin{cases} N_{wt-1}(1-\phi)+N_{dt-1}(1-\varepsilon)(r_t-r_{t-1}) & \text{if} \quad r_t>r_{t-1} \\ N_{dt-1}(1-\varepsilon)+N_{wt-1}(1-\phi)(r_t-r_{t-1}) & \text{if} \quad r_t<r_{t-1} \end{cases}$$ [7]

for the wet season. For the dry season,

$$n_{dt} = \begin{cases} N_{dt-1}(1-\varepsilon)+N_{wt-1}(1-\phi)(r_{t-1}-r_t) & \text{if} \quad r_{t-1}>r_t \\ N_{wt-1}(1-\phi)+N_{dt-1}(1-\varepsilon)(r_{t-1}-r_t) & \text{if} \quad r_{t-1}<r_t \end{cases}$$ [8]

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Similarly,

$$nn_{wt} = \begin{cases} N_{wt-2}(1-\mu)^2 + N_{dt-2}(1-\beta)^2(r_t - r_{t-1}) & \text{if} \quad r_t > r_{t-1} \\ N_{dt-2}(1-\beta)^2 + N_{wt-2}(1-\mu)^2(r_t - r_{t-1}) & \text{if} \quad r_t < r_{t-1} \end{cases} \qquad [9]$$

and

$$nn_{dt} = \begin{cases} N_{dt-2}(1-\beta)^2 + N_{wt-2}(1-\mu)^2(r_{t-1} - r_t) & \text{if} \quad r_t > r_{t-1} \\ N_{wt-2}(1-\mu)^2 + N_{dt-2}(1-\beta)^2(r_{t-1} - r_t) & \text{if} \quad r_t < r_{t-1} \end{cases} \qquad [10]$$

Reproductive adaptations

In some species (e.g. springbok, Jackson 1995), when a female's offspring die before being weaned, she may come into oestrus and mate. This is due to the removal of lactational anoestrus imposed by the suckling animal. Suppose that if females lose their neonates, they exhibit oestrus and are mated. If the favourable and unfavourable seasons are of equal length, such females will give birth in the opposite season to the one in which the neonate was lost. For instance, if a female loses her neonate and is re-mated midway through the wet season, she will give birth midway through the dry season. Thus, assuming that all females that lose offspring are re-mated in the same season, the number of re-mated females (δ) in the wet season is given by

$$\delta_{wt} = n^{\beta}_{dt-1} \qquad [11]$$

For the dry season

$$\delta_{dt} = n^{\mu}_{wt-1} \qquad [12]$$

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Case I: Obligatory reproduction

If the onset of oestrus after the death of the neonate occurs regardless of the prevailing season, it can be said to be obligatory. This adaptation is often termed opportunistic because—despite the fact that environmental conditions may not be optimal for reproduction—the onset of oestrus provides an animal with an opportunity to reproduce after losing a previous reproductive investment. The total number of animals alive in each season is the sum of the number of neonates and the number of adults. Thus, assuming a sex ratio of 1:1, the total number of neonates in a season (X) is given by

$$X_t = \frac{(n_t + nn_t)}{2} + \delta_t \qquad [13]$$

Case II: Facultative reproduction

The females of many highly restricted species do not come into oestrus outside a well defined "breeding season". For example, in ewe lambs in which puberty could be reached, this first oestrus is delayed until the appropriate time in autumn (Foster *et al.* 1988). Thus, there are periods during which females exhibit oestrus and other periods where oestrus is not exhibited even if environmental conditions are similar. While obligate breeders show oestrus regardless of the prevailing season, animals that do not show oestrus outside a breeding period can be said to be facultatively reproductively active. Assuming that all females are facultatively reproductive, no females will be mated or re-mated outside the breeding season. Thus, the number of births in each season in this paradigm (Y) is

$$Y_t = X_t - \delta_t \qquad [14]$$

Calculations

The following assumptions have been made: (*i*) all ewes that are not suckling any offspring are mated and all pregnant females give birth, (*ii*) the length of gestation is 6 months, (*iii*) all

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neonates that survive are fertile the following season, and (*iv*) the transition from one season to another is instantaneous and discrete. The model calculations are based on equations [7 – 14] and the definitions and conditions relevant to the model are summarised in Table 7. The equations can be iterated for any length of time (t)—that is, for any number of years—to determine the distribution of births in each season over time. Ideally, iterations should proceed until changes in the distribution of animals between the two seasons with time reaches some form of equilibrium. Equilibrium is here defined as the first point in time (in this case, that year) that satisfies the following two conditions: (*i*) the proportion of neonates born in the wet season is 1 and (*ii*) the mean proportion of neonates born in the wet season for the rest of the series is ≥ 0.95. Skew is simply defined as the relative proportion of neonates born in the wet season (the number of neonates born in the wet season divided by the total number born in both seasons at the equilibrium point). Thus, the maximum skew is 1 (all neonates born in the wet season) and the low skew is 0.5 (neonates are distributed equally in each season). The relevant equations were iterated 99 times (= 100 years). For these basic calculations, skew was set to 0.5 (i.e. equal distribution of animals between each season) and the values of the variables were set as follows: $\mu = 0.1$, $\beta = 0.15$, $\phi = 0.15$, $\varepsilon = 0.2$, $r = 0.5$, $N_{wt=0} = 100$, $N_{dt=0} = 100$. An example of the iterative calculation procedure is given in Box 3.

Subsequent to the basic model iterations, the effects of a randomly fluctuating wet season length (r) and the rate of mortality on the equilibrium parameters was assessed. Finally, the effect of reproduction adaptations on the equilibrium parameters (Case I and II, described above) was examined.

Results

Variability in the duration of the favourable season

If the length of the wet (favourable) season (r) is constant—irrespective of its value—the time to equilibrium is constant: the distribution of neonates in the wet season increases steadily until a stable equilibrium is reached where skew is maximal (Figure 32*a*). If r is variable, the equilibrium is not stable but is dynamic and the distribution of animals between each season

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can change rapidly (Figure 32*b* & *c*). As the value of *r* increases, the degree of skew at equilibrium decreases.

Environmental pressure

As the ratio of mortality of neonates in the wet season relative to neonatal mortality in the dry season (the ratio $\mu:\beta$) increases, skew decreases (Figure 33*a*). If mortality in both seasons increases, the time taken to reach equilibrium decreases (Figure 33*b*).

Reproductive adaptations

There was no difference in the results of the iterations irrespective of whether post-partum oestrus in individuals was assumed to be obligatory (Case I) or facultative (Case II). Thus, whether or not reproduction is timed had no quantitative or qualitative effects on the results.

Discussion

Interpretation of the model

The model makes the following predictions. First, the degree of skew should reflect environmental variability and differences in seasonal mortality rates. This prediction is corroborated by the fact that skew and environmental variability are weakly correlated in ungulates (see Chapter 3 and Appendix 2, p. 31 & 90).

Second, the model suggests that "timing adaptations", such as the pineal-melatonin system, are not a prerequisite for the development of highly skewed distributions. Thus, species in which the onset of reproduction is obligatory should still exhibit high skew in an appropriate environment, i.e. an environment with reasonably constant season lengths and/or different rates of environmental pressure between seasons. This prediction is difficult to corroborate because there are few examples of species that lack the basic physiological basis of timing adaptations (Goldman & Nelson 1993). However, in Chapter 4 and 5, it is suggested that

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springbok may not use the pineal-melatonin system (probably the most ubiquitous physiological timing adaptation, Arendt 1995), yet springbok populations in many northern and southern hemisphere localities are nevertheless highly skewed (see Figure 10 in Chapter 3, p. 34). Furthermore, animals that are non-photoperiodic are not uncommon in some populations of photoperiodic animals that are highly restricted breeders (Keller & Krebs 1970, Desjardins & Lopez 1983).

Third, the model predicts that species with timing adaptations should have low skew in an appropriate environment, i.e. an environment with variable season lengths and/or similar rates of environmental pressure between seasons. Thus, the existence of timing adaptations does not preclude the development of low skew.

Fourth, when the season length is highly variable and/or environmental pressure is markedly different between seasons, oestrus might be expected to occur after the cessation of lactational anoestrus (i.e. death of the neonate). That is, reproduction should be obligatory, particularly if selection has proceeded for long enough on that population. The reason is that opportunistic individuals that are able to use post-partum oestrus should, on average, have a greater fitness than individuals that cannot take advantage of this mechanism to increase their reproductive output. Indeed, many gazelles that live in environments with reasonably unpredictable seasons characterised by small differences in environmental pressure between seasons exhibit this behaviour, e.g. see Jackson (1995). In contrast, species from highly stable temperate environments with great differences in environmental pressure between seasons do not exhibit post-partum oestrus, i.e. they are facultatively reproductive; rather, the onset of reproduction is confined to a defined breeding season.

Finally, a logical corollary to the model predictions related above is that the reproductive behaviour of males should reflect that of the females. This is because the temporal distribution of sexually receptive females is an important determinant of the behaviour of males that attempt to maximise the number of possible matings (Ims 1987). Thus, where timing adaptations exist in females, males should opt for a conservative approach to mating: they should time their mating activities to coincide with the females. In contrast, when timing

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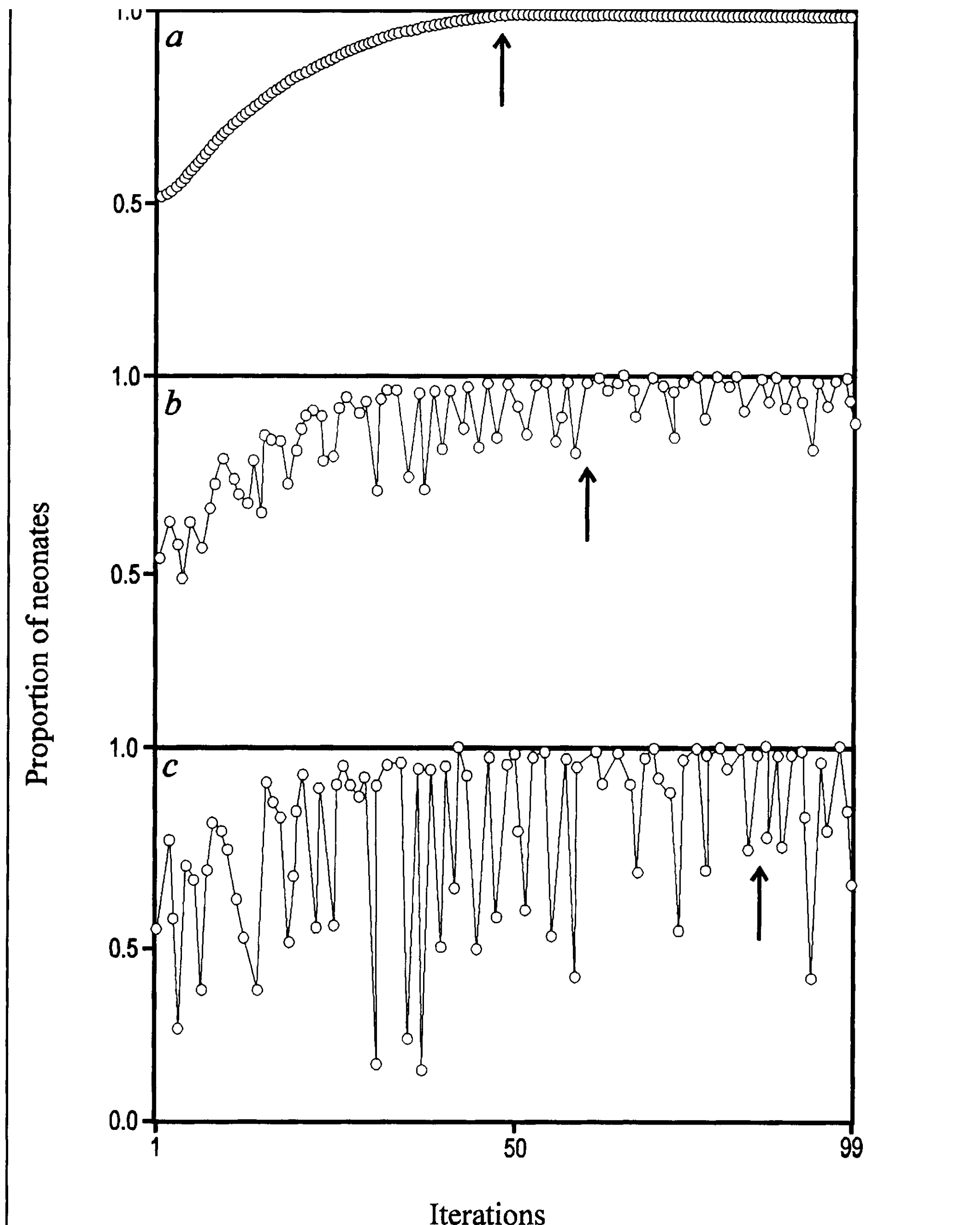

Figure 32. Results of the various model iterations.

(a) Results of basic iterations of the model equations. The figure shows the proportion of neonates in the wet season (closed circles) increasing over time (100 years). After initially being equally distributed between the wet and dry season (at time 0), the proportion of neonates in the wet season increases steadily until a static equilibrium is reached (indicated by the arrow). At equilibrium, the skew in the distribution of neonates is maximal (skew = 1).

(b) Results of iterations using the same values as in Figure 32*a*, except that the value of *r* has a random value between 0 and 0.33. As in Figure 32*a*, the proportion of neonates in the wet season increases over time. However, the equilibrium is not static but fluctuates within fairly narrow limits. Note that the equilibrium point (indicated by the arrow) is shifted to the right relative to that in Figure 32*a*.

(c) Results of iterations using the methodology of Figure 32*a* and *b*. Here, the value of *r* has a random value between 0 and 1. As in Figure 32*a* and *b*, the proportion of neonates in the wet season increases over time. At equilibrium, the distribution of neonates between the two seasons fluctuates randomly within far greater limits than the case in Figure 32*b*. The equilibrium point (arrow) is shifted further to the right.

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adaptations are absent (or masked), males should be opportunistic and remain reproductively active throughout the year. Hence, springbok rams in the southern Kalahari, where the populations are not highly skewed, are physiologically capable of reproduction throughout the year (Skinner & Van Zyl 1970, Skinner *et al.* 1996); on the other hand, impala rams have a marked sexual cycle with a peak in physiological mating capacity during the autumn rut that coincides with the highly restricted breeding period of the females (Anderson 1965, Skinner 1971).

The effect of variability in season length and environmental pressure on the establishment and maintenance of skew

The results of the model presented here show that in an environment with two distinct seasons, the lengths of which are reasonably constant and hence predictable, selection will produce a skewed annual distribution of births with variable stability. Similarly, if the seasons are characterised by different rates of environmental pressure, skew will develop. The degree of skew is determined by the relative variability of the season length and the relative difference between seasonal environmental pressure, although the former factor is of overriding importance. For instance, Rutberg (1984) concluded that climatic factors (represented by "variability in season length" in the model presented above), rather than Estes' (1976) "predator swamping" hypothesis (comprising "environmental pressure" in the model), best explains the highly skewed distributions of births in bison populations. Skew is lowest where season lengths are unpredictable and environmental pressure great. This is intuitively apparent: the occurrence of favourable conditions is difficult to predict when the length of a season is variable and births often occur outside the optimal period, purely by chance. If this is compounded with high environmental pressure in a season, the result is a highly skewed birth distribution. However, when the absolute seasonal rates of mortality are low, selective pressure for skew is low: animals that produce offspring outside the favourable period are not penalised as heavily as when mortality is great. Hence, the existence of animals in the unfavourable period can persist and result in low skew.

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Timing of reproduction and reproductive skew

As individual fitness depends in part on the number of surviving offspring produced, adaptations that maximise reproductive output confer a selective advantage on individuals. Thus, animals that are able to restrict the birth of their offspring to the most favourable season (facultative reproductive adaptations) should, on average, be more successful than individuals that give birth outside this period. This is most relevant in an environment that is highly predictable. Selection should thus favour adaptations that allow reproduction to be restricted to the most appropriate time. One means of achieving this is by using environmental cues or *zeitgebers* to achieve coincidence of births and favourable environmental conditions. The results of the model, however, show that timing reproduction has no qualitative or quantitative effects on the degree of skew that develops in a population.

In contrast, in habitats that are highly variable, adaptations that maximise reproductive output without taking timing into account (obligatory or opportunistic reproductive adaptations) assume greater importance. This is because an animal in a variable environment that is re-mated whenever possible has a greater probability of successfully raising at least some offspring (i.e. greater fecundity) compared to an animal in the same environment that only reproduces at a set time of year. A similar situation exists when there is little difference in seasonal conditions. If the probability of success is not markedly different between seasons, an animal can balance the risk of mortality with the benefits of gaining additional reproductive output. This is obviously not the case when seasons differ markedly, where any mis-timed reproduction is highly unprofitable.

Conclusion

The model presented here suggests that timing devices are not an essential fitness component in all environments. Specifically, timing adaptations should only be effective in a highly stable environment with highly differential seasonal rates of mortality. Certainly, timing adaptations are a crucial fitness component of the *individual*; for instance, physically (Bittman *et al.* 1985) and functionally (Karsch *et al.* 1989) pinealectomised sheep fail to reproduce at the appropriate

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time. If this were the case in the natural environment, such individuals would undoubtedly have a relatively lower fitness than individuals with timing adaptations. Thus, selection should retain timing adaptations in an appropriate environment. Selection for control is weakest when there is little or no difference between mortality in each season, when the absolute mortality rates are low, and when there is great environmental variability (Figure 33). Thus, in such habitats, there is little selection for a controlled, facultative reproductive adaptation. In contrast, selection is strongest in stable habitats with high mortality that differs markedly between seasons. Perhaps this is why temperate species have timing devices such as the pineal-melatonin system and control reproduction through restricting oestrus to an appropriate time, and species in unpredictable habitats seem to be non-photoperiodic and able to reproduce throughout the year.

Furthermore, populations that are not skewed can develop irrespective of whether timing adaptations exist in the population. This is due to the relative decrease in the selective

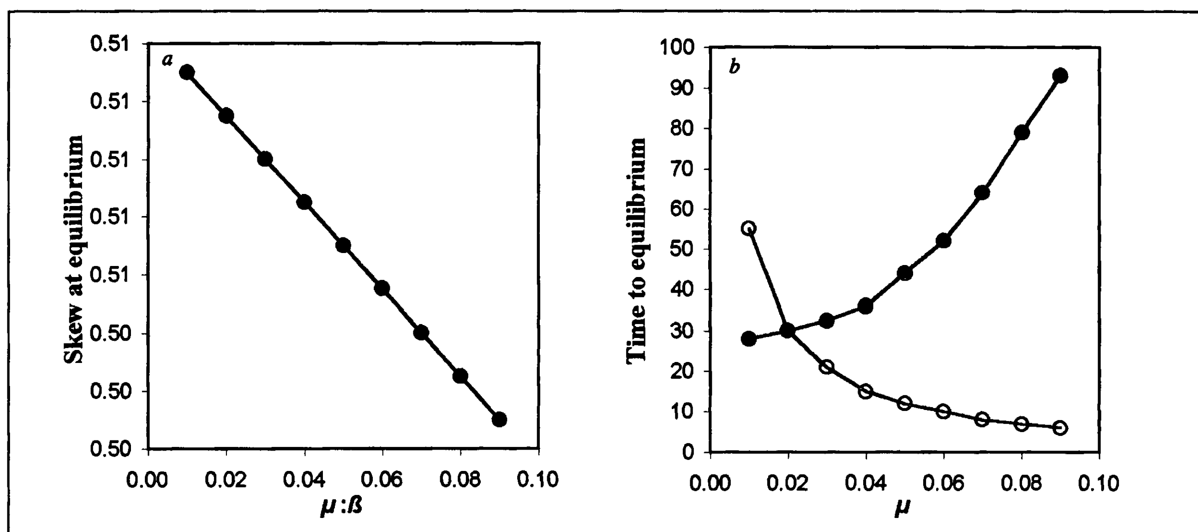

Figure 33. Summary of the main effects of the basic model.

(a) The effect of the ratio $\mu{:}\beta$ on skew at equilibrium. As the ratio $\mu{:}\beta$ increases, so skew decreases. As μ represents neonate mortality in the wet season, the figure shows that as the difference in mortality in the wet and dry season diminishes (i.e. as $\mu{:}\beta$ approaches unity), so the difference in the distribution of neonates in each season at equilibrium decreases.

(b) The effect of mortality on the time taken to reach the equilibrium distribution. As mortality in the wet season increases (the value of μ increases while all other values remain constant), it takes longer for the equilibrium to be reached (closed circles). In contrast, as absolute mortality increases (the values of the mortality coefficients increases equally in both seasons), the time to equilibrium decreases (open circles).

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advantage of animals with timing adaptations over animals without such adaptations as season length becomes variable and discrepancies in seasonal environmental pressure decrease (Figure 33*a*). Thus, in a habitat that is highly variable and/or has no marked differences between seasons, species should have low skew irrespective of whether timing adaptations exist. Sheep translocated to the equator exemplifies this: whereas they are highly skewed in the Northern Hemisphere, they are not generally skewed at the equator, despite the existence of timing adaptations in the animals (Sadlier 1969). The reciprocal of the above-mentioned phenomenon is that highly skewed populations can also develop irrespective of whether timing adaptations exist in the population.

Finally, the results of this Appendix demonstrate that the pineal-melatonin system is only useful as long as favourable environmental conditions can be reliably predicted, as originally noted by Negus & Berger (1972). When long-term *zeitgebers* that rely on stability are undermined by environmental variability, alternative cues to reproduction assume greater importance. For instance, many rodent species that are normally photoperiodic also respond in the short term to secondary plant compounds, such as 6-methoxy-2-benzoxazolinine (6-MBOA), which are short-term environmental *zeitgebers*. Thus, while maintained under a photoperiod that normally inhibits reproduction, 6-MBOA overrides photoperiod and stimulates reproduction in several species (Negus & Pinter 1966, Schadler *et al.* 1988).

Box 3. Example of the iterative calculation procedure used to develop the model. Values used: $r = 0.5$, $\mu = 0.05$, $\beta = 0.1$, $\phi = 0.005$, $\varepsilon = 0.2$, $N_{wt=0} = 10^3$, $N_{dt=0} = 10^3$.

	n_w	n_d	nn_w	nn_d	N_w	N_d	δ_w	δ_d	X_w	X_d	$\frac{Xw}{Xw + Xd}$
t_0					1000	1000					
t_1					995	800					
t_2	990	640	903	810	990	640	1.95	1.41	948	726	0.566224
t_3	985	512	898	648	985	512	1.91	1.41	943	581	0.618709
⋮	⋮	⋮	⋮	⋮	⋮	⋮	⋮	⋮	⋮	⋮	⋮
t_{99}	609	0	555	0	609	0	0.22	1.38	582	1.38	0.997638

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Table 7. Definitions and conditions of the model parameters.

	Definition	Conditions
μ	Mortality of neonates born in the wet season until their first birth	$0 < \mu < 1$ $\mu \leq \beta$
β	Mortality of neonates born in the dry season until their first birth	$0 < \beta < 1$ $\beta \geq \mu$
ϕ	Proportion of adult females born in the wet season that die each year	$0 < \phi < 1$ $\phi < \mu$
ε	Proportion of adult females born in the dry season that die each year	$0 < \varepsilon < 1$ $\varepsilon < \beta$
n	Number of offspring born to adult females	$n \geq 0$
nn	Number of offspring born to wet season neonates	$nn \geq 0$
N	Number of adult females	$N \geq 0$
r	Duration of wet season	$0 < r < 1$
δ	Number of offspring born as a result of mating in the "normal" season due to interruption of lactation anoestrus	$0 < \delta < 1$

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Summary

I addressed the phenomenon of aseasonal reproduction in ungulates using the springbok (*Antidorcas marsupialis*) as a model species. To determine whether reproduction in springbok is regulated according to environmental variables, I analysed birth data for wild and captive springbok populations from different localities. The degree of restriction of births could not be correlated to temperature, rainfall or latitude, which suggests that springbok do not use environmental *zeitgebers* to cue reproduction.

I investigated whether a group of springbok ewes kept in controlled, favorable conditions would have reproductive characteristics of seasonal or aseasonal breeders. To this end, blood was sampled and body weight obtained twice weekly. A radioimmunoassay was used to analyse progesterone secretion in order to elucidate the onset of puberty and describe the oestrous cycle. All of the study animals ovulated spontaneously at the onset of puberty (indicated by the first ovulation). The commencement of cyclic activity was not related to body weight in any obvious way, although there may be a minimum weight threshold that must be exceeded before cyclic activity can commence. While the fact that the animals did not attain puberty at the same time is a characteristic of aseasonal breeders, 80 % of the animals attained puberty within three months of each other, which suggests some seasonal influence. Thus, while springbok do not appear to be typical seasonal breeders, they are not be entirely aseasonal given an appropriate environment.

I investigated the pineal-melatonin system of the springbok by obtaining a series of jugular blood samples from captive animals during the day and at night. The concentration of melatonin in the blood plasma was determined by radioimmunoassay in order to elucidate the melatonin secretion profile of the animals. The results show that the springbok pineal-melatonin system is present and functions in the same way as in photoperiodic species. This suggests that this species "ignores" photoperiodic information that could be used to cue reproduction.

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The transition from reproductive quiescence to activity in female mammals was investigated using the sheep (*Ovis aries*) as a model species. A novel method of addressing the LHRH neurosecretory system via the third ventricle was employed. The practicability of this method was assessed by sampling ventricular CSF and jugular blood to analyse the secretion of LH (in blood), LHRH and selected neurotransmitters (in CSF). LH and LHRH were determined using radioimmunoassays while HPLC was used to determine the concentration of neurotransmitters. CSF and blood were collected from ewes intensively for six hours in a low (photo-inhibited) and high (photo-stimulated) pulsatile situation. The transition from the low to high pulsatile situation was monitored by obtaining CSF and blood samples twice weekly. The method proved to be less successful than anticipated and consequently the data that were collected must be interpreted with caution. Nevertheless, the results suggest that neurotransmitters have differential roles that are determined by the reproductive state of an animal and support the contention that there is a complex system of interneurones that link melatonin to the modulation of gonadotrophin secretion.

In order to assess whether xylazine could be used as a means of chemical restraint to study reproductive parameters in springbok, I investigated the effect of xylazine on LH secretion in ewes. The results showed that xylazine suppresses LH secretion and should thus not be used to restrain animals in which accurate assessments of centrally released hormones are required.

In order to clarify the concepts of seasonal and restricted breeding, I introduced a dimensionless index of restriction which I used, first, to quantify the degree of birth restriction in the population analyses throughout my thesis, and second, to illustrate the effect of phylogenetic constraints on reproduction.

Finally, I developed a model of the manner in which the temporal distribution of births is developed and maintained in populations. The model suggests that restricted breeding can develop in an appropriate environment regardless of whether animals in a population can time reproduction. Furthermore, non-restricted or aseasonal breeding is suggested to be a consequence of the environment rather that an inability to time reproduction.

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Opsomming

Ek het nie-seisoenale voortplanting by hoefdiere ondersoek deur die springbok (*Antidorcas marsupialis*) as model te gebruik. Om vas te stel of voortplanting in die springbok deur omgewingveranderlikes gereguleer word het ek geboortedata van wilde en aangehoude springbokbevolkings vanaf verskeie gebiede ontleed. Die mate waartoe geboortes beperk is, kon nie met temperatuur, reënval of geografiese ligging gekorreleer word nie, wat aandui dat springbokke nie van omgewingseine gebruik maak om hulle voortplanting te reguleer nie.

Ek het 'n groep springbokke aangehou om vas te stel of hulle enige van die voortplantingeienskappe van seisoenale voortplanters toon. Ek het twee keer per week bloedmonsters geneem en die liggaamsmassa van die diere gemeet. Plasma progesteroon konsentrasies is deur middel van radio-immuno-essaiering ontleed om die aanvang van puberteit te ontleed en om die estrussiklus te beskryf. Alle diere het spontaan begin ovuleer toe hulle puberteit bereik het (soos deur die eerste ovulasie aangetoon). Die aanbreek van puberteit is nie met ligaamsmassa gekorreleer nie, maar daar bestaan heel waarskynlik 'n kritieke massa-drumpel wat diere moet oorskry voordat ovulasie kan plaasvind. Alhoewel die diere nie almal, soos wat normaalweg 'n aanduiding van nie-seisoenale voortplanting is, terselfdertyd puberteit bereik het nie, het 80 % van hulle puberteit binne drie maande van mekaar bereik. Dit dui op 'n beperkte mate van seisoenale voortplantingsgedrag. Terwyl springbokke nie kenmerkend seisoenale telers is nie, het hulle wel nie al die voortplantingeienskappe van seisoenale voortplanters getoon nie, en is hulle nogtans nie heeltemal nie-seisonaal in 'n geskikte omgewing nie.

Ek het die pineale melatonien sisteem van springbokke ondersoek deur 'n reeks bloedmonsters op verskeie tye van die dag en nag te neem. Melatonien in die bloedplasma is deur middel van 'n radio-immuno-essaiering bepaal sodat die melatonien uitskeidingsprofiel van die diere verklaar kon word. Die resultate het bewys dat die pineale melatonien sisteem van springbokke wel bestaan en op dieselfde wyse funksioneer as by fotoperiodiese diere. Dit wil voorkom as of springbokke fotoperiodiese inligting, wat vir voortplantingsregulering gebruik kan word, ignoreer.

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Die veranderinge in vroulike soogdiere wat vanaf 'n onaktiewe na aktiewe voortplantingstatus verskuif is ondersoek deur skape *(Ovis aries)* as 'n modelspesie te gebruik. 'n Nuwe metode om die LHRH uitskeidingsisteem via die derde ventrikel te ondersoek, is gebruik. Die bruikbaarheid van hierdie metode is bepaal deur monsters van ventrikale-CSF en nekaarbloed te neem om die uitskeiding van LH (in bloed), LHRH en geselekteerde neurotransmitters (in CSF) te ontleed. LH en LHRH is deur middel van radio-immuno-essaiering gekwantifiseer, terwyl HPLC gebruik is om die neurotransmitters te kwantifiseer. CSF en bloed is van ooie getrek tydens 'n intensiewe tydperk van 6 ure in omstandighede van hoë en lae uitskeidingsfrekwensie. Die oorgang van 'n lae na 'n hoë uitskeidingsfrekwensie is gemonitor deur CSF en bloed twee keer per week te trek. Hierdie metode was uiteindelik minder suksesvol as wat verwag is, en daarom moet die data wat versamel is met omsigtigheid geïnterpreteer word. Desnieteenstaande dui die resultate aan dat neurotransmitters verskillende rolle het wat deur die voortplantingtoestand van die dier bepaal word. Dit onderskryf die standpunt dat daar 'n skakel tussen die uitskeiding van melatonien en voortplantingshormone, in 'n komplekse sisteem van interneurons, bestaan.

Om vas te stel of die verdowingsmiddel xylazine gebruik kan word om springbokke in bedwang te hou, het ek die effek daarvan op LH-uitskeiding in ooie ondersoek. Die resultate het aangedui dat xylazine LH-uitskeiding onderdruk. Xylazine behoort dus nie gebruik te word om diere in bedwang te hou nie indien die uitskeiding van sentraalgereguleerde hormone ondersoek word.

Om die konsepte van seisoenale en beperkte voortplanting te verklaar het ek 'n dimensielose indeks van geboortebeperking ontwerp en dit gebruik om eerstens, die omvang van geboortebeperking te ontleed en tweedens, om die effek van filogenetiese beperkings op voortplanting te illustreer.

Laastens het ek 'n model opgestel van die wyse waarop temporele verspreiding van geboortes in bevolkings ontwikkel en onderhou word. Die model dui aan dat beperkte voortplanting altyd in 'n geskikte omgewing sal ontwikkel, afgesien van die diere se tydsberekening van voortplanting. Verder word dit voorgehou dat nie-seisoenale of onbeperkte voortplanting die gevolg is van die omgewing eerder as die vermoë om voortplanting te tydsbereken.

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